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PREFACE

In the preface to the preceding volume we touched upon the comma, the full stop, the split infinitive, the obscure abbreviation, and jargon. With this as an introduction we consider it fitting to draw attention to other more important aspects of writing. Though we recognize that rules of punctuation, of spelling, and of sentence structure undergo their own evolutionary processes, we have found to our interest that changes wrought by the centuries in the use of words by the learned are not always an improvement: some of the wisest and saltiest bits of advice are to be found in publications which have rested in our libraries for many decades.

It seemed not inappropriate to turn to an early volume of a Review for illumination on this subject. Selection was made, not of an annual but a weekly Review,[1] the product of a much more industrious pen. In a review of a small tract[2] the author proceeds:

> He thinks the world cloy'd with many trivial and useless writers, but gives this as a rule: If men would take care that ill Books be not written, and that good Books be not ill written; but that in their composing a due regard be always had of *Prudence*, *Solidity*, *Perspicuity*, and *Brevity* there would be no cause left for us to complain of the too great number of Books.
>
> *Prudence* chiefly consists in this, that a man never apply himself too rashly or inconsiderately to write, but that he first learn and well imbibe what he purposes to teach others. . . .
>
> So he says men should be no less studious of *Solidity;* (he means in Subjects that will bear it, not in things written more for the diversion of the Mind than for correctness) that is, men must take care that what they write be not weak, frivolous, doubtful, carrying little shews of truth; but things that are true indeed, firm, and beyond all exception; and so fortified and confirmed with stress of Argument, that they manifestly shew labour, and readily force the assent of *Readers*. Not that this *Solidity* can be everywhere observed alike, it being above the infirmity of man so to do; but men should be very wary not to flatter themselves so far, as to think that others are bound to believe their bare say-so's.
>
> He requires also such *Perspicuity*, that he would have the *Book* which is made publick to be the Looking-glass of the Author's mind; clearly presenting the sence of what is written to the *Reader*. As for Authors who use an affected obscurity in their Writings, as though they desired not to be understood, he thinks they might gratifie their own humour and the World much better by being silent.
>
> Lastly, he will not have a man so far indulge *Perspicuity*, as to neglect a due Brevity; For as *Obscurity* makes a Book useless, so if drawn out in *Length*, it becomes tedious. . . .

[1] Weekly Memorials for the Ingenious: or, an Account of Books lately set forth in several Languages. With other Accounts relating to Arts and Sciences. Printed for Henry Fairborne and John Kirsey, at the Rose in St. Paul's Churchyard, London, 1683.

[2] Christiani Liberii, Germani, Bibliotechos, Sive de scribendis legendis & aestimandis libris Exercitatio Paraenetica, & Ultrajecti. in 12° 1681.

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We cannot claim, nor would the authors, that everything in the pages that follow displays the perfection that would ensue from the exercise of Prudence, Solidity, Perspicuity, and Brevity. We believe, however, that the authors have made an heroic attempt. We trust that their readers will appreciate the difficulty of the task and will join with us in expressing their gratitude. Some of us desire that the reviews shall be selective but critical, judicious appraisals of the present status of the subject; others, fortunately in the minority, request that they be comprehensive with every paper on the subject receiving citation.

Our very warm thanks are extended to our editorial assistants and their associates in the business office, all of whom have had much to do with the present volume. Mrs. Lillian Rutherford has been the principal editorial assistant for *Biochemistry* while Dr. Donald Kupke gave us invaluable aid in preparing the subject index. As always we are indebted to our printers, the George Banta Publishing Co., for their complete co-operation.

H.J.A.	T.H.J.
H.J.D.	H.S.L.
W.Z.H.	J.M.L.
B.L.H.	G.M.

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page 155, lines 1 and 2: *delete* ribose-5-P
page 226, line 16: *for* Gorton *read* Garton
page 241, reference 180: *for* Gorton, G. A. *read*, Garton, G. A.
page 339, reference 23: *for* Iacobellis, N. *read* Iacobellis, M.
page 339, reference 23: *for J. Dairy Sci. read J. Nutrition*

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page 596: *insert* 226 *after* Garton, G. A.
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HERMAN AUGUSTUS SPOEHR
1885–1954

HERMAN AUGUSTUS SPOEHR
1885–1954

Herman Augustus Spoehr was born in Chicago, Illinois, on June 18 1885. He was the son of Charles A. and Frida Baeuerlen Spoehr. Although both his parents were natives of Goeppingen, Wuerttemberg, they met in America.

Spoehr's father came to Chicago about 1868, just before the Chicago Fire which deprived him of most of his worldly possessions. After the fire, he established a candy manufacturing business which grew successfully, along with the great burgeoning mid-west metropolis, to become one of the outstanding enterprises of its kind in the country. The interests of the candy business were logically expanded into the manufacture of sugar and chocolate, the latter of which Spoehr's father pioneered in this country.

Spoehr received most of his early schooling at private schools in Chicago. His secondary-school training was obtained at Lewis Institute, and his college work was taken at the University of Chicago, from which he received his baccalaureate degree in 1906. The year after graduation from college was spent in Europe studying with the two most noted European carbohydrate chemists of his time, Emil Fischer of Berlin and L. Maquenne of Paris. Following his sojourn in Europe, he returned to his Alma Mater for further training; there he received his Doctor of Philosophy degree in 1909. At Chicago he worked with another eminent carbohydrate chemist, John Ulric Nef—one of the great company of scholars which had been attracted to the newly founded university.

One could hardly be interested in sugar chemistry without wondering about the origin of sugar in nature. In order to prepare himself to satisfy this curiosity, Spoehr elected botany as his secondary subject and studied Plant Physiology with Charles Reid Barnes. According to Mrs. Spoehr, he "encountered considerable disparagement from some of the chemistry faculty for such an 'impossible' combination." But it was this "impossible combination" which formed the foundation for most of Spoehr's scientific work.

In a fashion characteristic of his forebears, Spoehr left the familiar surroundings of his youth to pioneer untried fields in a strange land. In 1910 he and his bride left the mid-west metropolis to establish their home in the small frontier town of Tucson, Territory of Arizona, he to be Chemical Plant Physiologist at the recently established Desert Laboratory of the fledgling Carnegie Institution of Washington. The ten years which he spent in the Arizona desert profoundly affected his scientific thought.

Except for the year 1930–1931, when he served as Director of Natural Sciences of the Rockefeller Foundation, Spoehr was an active member of the Carnegie Institution's staff from 1910 until he retired in 1950, being Chair-

man of the Division of Plant Biology from 1928 to 1947. Even after retirement he remained closely associated with the Department of Plant Biology until his death on June 21, 1954. Those of his immediate family who survive him are his widow, Florence Mann Spoehr, a son, Dr. Alexander Spoehr, and a daughter, Mrs. Horace P. Miller. The scientific pioneering so distinctive of the father is being continued by the son who is Director of the Bernice P. Bishop Museum of Honolulu.

Spoehr's scientific contributions were numerous and significant. They centered about one theme—photosynthesis. It is difficult now to realize the state of photosynthesis research at the time Spoehr began. No other laboratories in America were engaged in such research. In Europe, the classic work of Willstaetter and his collaborators, especially Stoll, was just getting under way. And in his early years Spoehr was possibly the sole link between this country and Europe in this important field.

Since he had a lively interest in carbohydrate chemistry and because carbohydrates are the principal products of photosynthesis, a large portion of his personal research was concerned with seeking to understand their reactions as they pertained to photosynthesis.

While at Tucson, Spoehr investigated the carbohydrates of the cacti and their transformations relative to the extreme changes encountered in the desert environment. This work culminated in a notable monograph, *The Carbohydrate Economy of the Cacti*. The diurnal fluctuations of the organic acids of the cactus plant and their reciprocal relation to the sugars also engaged his attention. An examination of the products obtained from the photolysis of these acids *in vitro* provided a partial explanation of the changes observed in the living plant. He identified formaldehyde among the photolysis products of the pure acids. Consequently, the presence of formaldehyde in leaves could no longer be considered sure evidence for the Baeyer formaldehyde theory of photosynthesis. This observation, in conjunction with his meticulous experiments on the photoreduction of carbon dioxide to formaldehyde which failed to substantiate the affirmative claims of others, greatly hastened the abandonment of the Baeyer theory.

After moving to the Carnegie Institution's Coastal Laboratory at Carmel, California, in 1920, Spoehr in collaboration with a number of co-workers continued his experiments on various phases of carbohydrate chemistry and photosynthesis. His study of the transformation of the sugars by purely chemical means fully demonstrated various interconversions of the trioses and hexoses among themselves and among each other under conditions of temperature and alkalinity (phosphate and carbonate buffers) commonly existent in biological systems.

The monograph, *Studies in Plant Respiration and Photosynthesis*, published jointly by Spoehr and J. M. McGee, emphasized the possible connection between these two processes and revealed new interrelationships between the sugars and amino acids in them. Spoehr was much interested in the mechanism of the biological oxidation of sugars. To study this, he invented

a catalytic procedure which simulated the biological process in that it could oxidize sugars to carbon dioxide with air at body temperature and at physiological pH values. The active catalyst in this process was sodium ferropyrophosphate.

Considerable effort was expended to find the "substance on to which carbon dioxide . . . can be added" in photosynthesis. The first experiments showed the carbon dioxide uptake to be predominantly ionic in nature, but some years later, through the use of radioactive carbon, C^{11}, an organic acceptor was demonstrated to exist in the leaf which took up relatively small quantities of this gas.

While still at Carmel, the series of researches on the pigment systems in photosynthetic organisms was begun. Initially, the work was limited to the structural relations of certain carotenoids. But this work, in the hands of H. H. Strain, was soon expanded to include a survey of the carotenoids and chlorophylls of a large number of plants. This survey demonstrated the complexity and diversity of the photosynthetic apparatus among the different groups of plants. It uncovered a host of hitherto unknown carotenoids and, most surprisingly, some new chlorophylls.

One of the most notable achievements of Spoehr in the years spent in Carmel was the completion and publication of his book, *Photosynthesis*. This was an authoritative account of what was known about the subject at the time. The book marked its author as the distinguished writer, scientist, and scholar that he was. It became the bible for a generation of workers in this field and had much to do with exciting the present general interest in this subject.

In 1928 various groups of plant scientists operating within the Carnegie Institution were combined for administrative purposes into the newly created Division of Plant Biology with Spoehr as chairman. During 1928 plans were perfected for a new central laboratory of the Division to be built on the campus of Stanford University, and in 1929 this building was occupied. Although Spoehr's administrative load reduced his own research activities, he always had experimental work of his own in progress. This work was devoted to the study of the complex carbohydrates in leaves—starch, cellulose, and uronic acids; the conditions required for the activity of leaf enzymes, particularly the amylases; the organic nutrition of albino plants by which he brought them to maturity; and the mass culture of algae. With H. W. Milner he demonstrated the determinative influence of culture conditions on the internal composition of algae. During World War II he, with his associates, obtained antibiotics from autotrophic organisms, particularly from Chlorella. Even though some of these researches were carried on in collaboration with others, Spoehr participated personnally in the experimental work in all of them.

In 1947, three years before retirement, Spoehr resigned the chairmanship of the Division of Plant Biology, to which position C. Stacy French was appointed. During the three years previous to retirement and the four

years following, he continued his work on the carbohydrates of photosynthetic organs, isolating and purifying substances whose occurrence had only been postulated by indirect evidence gained by methods which were oftentimes of doubtful validity. During this time, several publications came from his hand. Most of these dealt with some aspect of the large-scale culture of algae—a process of industrialized agriculture which in time may bring new types of practices into agriculture.

Besides his scientific papers, Spoehr published a series of philosophical essays which treated, in the main, various aspects of the relation between science and society.

In addition to his regular duties, he performed many useful services to science. He counseled wisely and his advice was sought by many leaders in science, industry, philanthropy, education, and government. Spoehr was accorded many honors and was placed in numerous positions of responsibility and trust. It is not the purpose of this tribute to review these, since they have been enumerated in other places. Only his relations and contributions to Annual Reviews, Inc. will be elaborated here.

Spoehr's actual association with Annual Reviews began in 1933 with his article *Chemical Aspects of Photosynthesis* in volume II. In 1940 he was elected to the Editorial Board of *Annual Review of Biochemistry* and to the Board of Directors of Annual Reviews, Inc. In 1946 he was elected president of the latter board, which office he held until his retirement from the board in December, 1952. He was happy to accept these appointments and to do whatever he could to promote the aims of Annual Reviews for he felt that this was "one of the thoroughly worth-while undertakings in science." How certainly the aims of Annual Reviews harmonized with his conception of what publication had accomplished in science can be seen in a passage from his essay *The Nature of Progress in Science:*

> Without the careful recording of experiences, their correlation, examination in the light of what is already known, sifting the essential from the irrelevant, etc., knowledge would not grow. This organization of its experiences and constant re-examination of the groundwork on which it is building has been an essential element of scientific endeavor. As a consequence it has built up a body of knowledge which has been of inestimable value for its further development. This body of knowledge is contained in a system of written records which . . . considering the staggering number of individual and minute observations and conclusions which it contains is remarkably simple and couched in a language which for the scientific workers is practically universal. . . . The rapid development of science would have been utterly impossible if it had not so organized its body of knowledge that the experiences of one were made available to all . . . that the great mass of experience of an army of workers [was] summarized, generalized and organized, and for all time made available for the guidance of succeeding generations.[1]

There are few men whose background, training, and experience could

[1] "The Nature of Progress in Science," *Elihu Root Lecture of Carnegie Institution of Washington*, p. 48 (1935).

have fitted them so well for this appointment as those of Spoehr. His business and administrative experience, his familiarity with Europe, acquired as a youth and graduate student there, his personal acquaintance with a large number of European scientists, and his first-hand knowledge of their work, gained through his investigation of European silviculture during 1926 and his year as Director of Natural Sciences of the Rockefeller Foundation, all were of great value to Annual Reviews.

Many of the years he served on these boards were decisive ones. The number of Annual Reviews increased from two to eight. This expansion required many critical decisions to be made. The launching of new Reviews created problems concerning publicity and finance, delineation of boundaries between Reviews covering overlapping fields, selection of editors and editorial boards, and a host of minor matters relating to policy and operation.

World War II and the reconstruction period which followed imposed great strain upon an organization such as Annual Reviews which operates on an international scale and in an international atmosphere. With half the world beyond reach, the broad base of authorship on which Reviews depends was greatly curtailed. Even more serious was the restricted access to much of the research being done both in Europe and in America.

Business decisions were also numerous and burdensome. Many foreign outlets for the Reviews were temporarily stopped. With the cessation of hostilities would come a flood of requests to fill the gap caused by the war—but how many volumes would be wanted and how long must the extra inventory be kept? In the reconstruction period the need for these Reviews was great but the money to pay was limited. In how far could the largess of Annual Reviews be stretched? To solve these problems and to answer these questions successfully required thought, experience, and good judgment.

An appreciation of Spoehr's contribution to the successful handling of these matters is given by the editor of this Review in his letter regretting Spoehr's retirement from the board: "You have been with the enterprise for many years, during most of which you steered the ship and brought us safely around some of the treacherous shoals which had to be navigated. I sincerely hope that we may continue to have your advice from time to time on matters that call for your own good judgment and experience."

In his contacts with scientists from home and abroad during his service as scientific consultant to the Secretary of State in 1950, Spoehr was gratified by the frequent mention of Annual Reviews. He was impressed by what a powerful instrument these Reviews could become for cultivating international good will and understanding.

Spoehr's accomplishments as an eminent scientist, writer, and administrator give only a fragmentary picture of the full life he lived. Outside his professional activities, he had varied and deep interests in business, civic, and cultural affairs. Acquaintance with the real warmth of his personality, his charitableness, generosity, and magnanimity came only gradually

through long and friendly association with him. He was a lover of beauty wherever it was found: in art, literature, music, science, nature, nobleness of character, or in social relationships. He sought always to surround himself with it in his home, his garden, and his laboratory.

Perhaps no better way to close this tribute to our valued colleague and friend could be found than to quote the verse he himself used when relinquishing the chairmanship of the Division of Plant Biology:

> "The race of man is as the race of leaves:
> Of leaves, one generation by the wind
> Is scattered on the earth; another soon
> In spring's luxuriant verdure bursts to light.
> So with our race; these flourish, those decay."[2]

JAMES H. C. SMITH
C. STACY FRENCH

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[2] *Homer's Iliad*, Book VI, Line 171 (Edward Earl of Derby, Trans.).

BIOLOGICAL OXIDATIONS[1,2]

By David E. Green and Helmut Beinert

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The subject of biological oxidations encompasses a great many diverse areas of investigative activity. It seemed impossible to us to do justice to all the individual contributions within the allotted space. As a solution to the dilemma we have elected to concentrate on those lines of experimental evidence and interpretation which point to new concepts and developments in the major areas of the field. Furthermore it seemed to us redundant to report in this review material which had already been covered authoritatively in more detail elsewhere. The attention of the reader will be directed to these reviews as the occasions arise.[3]

The Mitochondrial System

The systematic study of the mitochondrion (defined here in a functional rather than a morphological sense) and its derivative enzymes has led during the past five years to some of the most important advances in our knowledge of enzymatic mechanisms. In view of the rapidity and broad scope of these advances we have felt there is need at present to review in some detail the literature bearing on the enzymatic properties of the mitochondrial system with an eye to defining the areas both of agreement and uncertainty. The comprehensive monograph by Lindberg & Ernster (2), the recent symposium on the structure and biochemistry of mitochondria (3), and some earlier reviews (4 to 8) are pertinent to this subject.

When prepared from the same source under exacting conditions the mitochondrion exhibits a uniform metabolic spectrum and a constant activity for any given enzymatic function. This conclusion can be drawn from the reproducibility of experiments with mitochondrial systems and specifically of those on oxidative phosphorylation (9, 10, 11), a process which probably involves a great variety of enzyme systems. Such evidence may be taken to mean that the composition and proportions of the several hundred enzymes and their respective coenzymes which are present in each mitochondrion are essentially constant providing the method of preparation is rigidly con-

[1] This review covers the literature up to approximately November, 1954.

[2] The following abbreviations will be used: AMP for adenosine-5′-monophosphate; ADP, GDP, and IDP for the 5′-diphosphates and ATP, GTP, and ITP for the 5′-triphosphates of adenosine, guanosine, and inosine, respectively; DPN for diphosphopyridine nucleotide; TPN for triphosphopyridine nucleotide; DPNH for reduced DPN; TPNH for reduced TPN; FAD for flavin-adenine dinucleotide; FMN for flavin mononucleotide; $FMNH_2$ for reduced FMN; CoA for coenzyme A; and DNP for 2,4-dinitrophenol.

[3] A critical and comprehensive review of oxidative enzymes, exclusive of hemoproteins, has recently been presented by Singer & Kearney (1).

trolled. One could then conceive of the mitochondrion not as a mixture of enzymes both soluble and particulate which are linked or complexed together in random and nonuniform proportions but as a precise, organized system of enzymes hardly different from a single enzyme in respect to uniformity but vastly different in respect to size, complexity, and the number of catalytic functions.

Assuming that a typical rod shaped mitochondrion is 3 μ in length and 0.5 μ in diameter (cf. 3) one can calculate how many molecules of a particular enzyme, e.g., α-ketoglutaric dehydrogenase, are present in each mitochondrion on the basis of the experimentally determined ratio: α-ketoglutaric dehydrogenase activity per mitochondrion to activity per molecule of dehydrogenase.[4] According to such a calculation from the data of Maley & Plaut (11) and Sanadi & Littlefield (12), there are some 10^3 molecules of α-ketoglutaric dehydrogenase per mitochondrion, a number which permits the conclusion that the mitochondrion is a structure in which some basic enzymatic sub-unit is repeated a large number of times. Such a sub-unit, of course, may contain more than one molecule of a given enzyme. Furthermore there may be more than one type of repeating unit.

An extensive effort has been aimed at determining whether a particular enzyme is localized in or outside the mitochondrion by quantitative assay of enzymatic activity in different cell particulates and in the aqueous medium of cell homogenates (5). Although these studies have yielded a large volume of information they have not led to unambiguous interpretations. For example the conclusion (13) that the isocitric dehydrogenase is not localized in the mitochondrion, although the mitochondrion is supposed to be the seat of the citric acid cycle, hardly leads to a plausible picture of the enzymatic pattern of mitochondria. Quantitative determinations of enzymatic activity in different cell fractions are beset with many difficulties (cf. 14). Unless they are carried out with full appreciation of the factors involved in multienzyme sequences and of the conditions necessary for measuring the activity of mitochondrial enzymes, questions of localization are not easy to decide. There appears to be no known exception to the rule that when the intact mitochondrion carries out some metabolic sequence such as fatty acid oxidation or the citric acid cycle all the constituent enzymes are found in the mitochondrion and no external supplementation is necessary. This is not to gainsay the fact that some mitochondrial enzymes may also have their counterparts outside the mitochondrion.

Although so far in this review the individual unit of oxidative activities in the cell has been treated as the "mitochondrion" the question of the heterogeneity of mitochondria is by no means a closed one. We may first

[4] In this calculation the assumption is made that the activity of the isolated enzyme is not vastly different from that of the intramitochondrial enzyme. According to this calculation α-ketoglutaric dehydrogenase contributes about 4 per cent of the mitochondrial protein.

consider differences between mitochondria from different organs or forms of life.

Mitochondria from animal tissues uniformly carry out the first three of the following reaction sequences: (*a*) the citric acid cycle, (*b*) oxidative phosphorylation, (*c*) electron transport between DPNH[2] or succinate and molecular oxygen by way of the cytochrome system and (*d*) fatty acid oxidation. The last sequence has not yet been demonstrated unambiguously in brain mitochondria (15). There are other major sequences such as the reactions of C_1 units (serine to glycine) (16) and reactions of the urea cycle (ornithine to citrulline) (17, 18) which may apply only to mitochondria from liver and kidney. In a general way it may be stated that mitochondria regardless of source carry out the citric acid cycle and related processes such as oxidative phosphorylation and electron transport. Then in addition to the basic common apparatus each mitochondrion exhibits specific metabolic characteristics by which the unit of one cell type can readily be distinguished from that of another. Thus rabbit liver mitochondria can oxidize propionate whilst rabbit kidney mitochondria cannot (19). Plant mitochondria (20, 21) also have their own distinctive pattern which sets them off from their counterparts in animal tissues. There is also evidence of considerable variation in size in the case of plant and bacterial mitochondria (4, 22).

Secondly, it is not easy to decide experimentally whether a suspension of mitochondria from one type of cell is a mixture of particle species each with a substantially different array of enzymes. Such a mixture would be hard to reconcile with the interrelationships known to exist between the different reaction sequences characteristic of mitochondria. If sequence A were carried out in particle type A and sequence B in particle type B then some device would have to exist to couple the events in particle A with those of particle B. There is no evidence either for the presence of or requirement for such coupling agents.

Kuff & Schneider (23) have compared the ratio of four enzymatic activities in different layers of sedimenting mitochondria from mouse liver. Differences in the ratio were observed. Similar results were reported by Paigen for rat liver mitochondria (24) and by DeDuve *et al.* (25) who have interpreted these differences in enzyme ratios in terms of different particle types. One would like to see conclusions as to the possible existence of mitochondria with basically different metabolic spectra drawn on the basis of studies of main sequence enzymes rather than of peripheral enzymes such as phosphatase or uricase.

When mitochondria are exposed to the shearing forces of the rapidly rotating blade of a blendor or to sonic vibrations the structural units are disrupted with liberation of soluble and particulate elements. The relative amounts of these two elements are not constant and are greatly influenced by a variety of factors such as the time and speed of blending, the pH, and the ionic strength of the suspension medium (26, 27, 28). Much evidence has been accumulated which points to changes in state of some enzymes from

particulate to soluble form. DPNH cytochrome-*c* reductase (29)[5] and succinic dehydrogenase (30, 31) are two unambiguous examples of enzyme activities which are exhibited both by particulate and soluble entities. It is accordingly difficult to extrapolate back to the mitochondrion and assess what proportion of the mitochondrial protein is particle-bound on the basis of the proportion found to be soluble in the extracts of disrupted mitochondria.

The application of phase and electron microscopy to the examination of mitochondria has provided valuable information on the internal structure and arrangement of the mitochondrion. On the basis of his studies with the phase microscope, Harman (32) drew attention to the existence of differentiated areas within damaged mitochondria and correlated changes in the dense area (crescent) with loss of activity. Palade (33) has examined the fine structure of sectioned animal mitochondria in the electron microscope and found a characteristic pattern of organization irrespective of the species of origin and the cell type. Similar studies have been made by Sjöstrand & Rhodin (34, 35) and by Weinreb & Harman (36, 37). Although there is conflicting evidence on certain points which will have to be resolved by further work it is clear beyond doubt that mitochondria have a highly organized and regular internal structure. A great number of fine lamellae[6] are visible which seem to extend in a very regular fashion partly (33) or all the way (36) through the mitochondrial body in a plane perpendicular to the long axis of the mitochondrion. These lamellae have been regarded as the seat of the structure-bound enzymes while the protein matrix constituting the rest of the mitochondrion has been visualized as the seat of the so-called soluble enzymes. The question whether mitochondria possess a boundary layer with the characteristics of a membrane is as yet not resolved. Although the electron micrographs of Pallade (33) and of Sjöstrand (34, 35) lend themselves to the interpretation that a membrane actually exists, comparable pictures of Weinreb & Harman (36, 37) and of Powers *et al.* (38) do not show a boundary which could be identified as a membrane. There is a difference of opinion as to the nature of the interior of the mitochondrion in which the network of lamellae is found. According to Pallade the interlammelar space is fluid whereas according to Harman the interlammelar space is filled by a continuous gel. As to the basic principles which the enzymologists require in order to construct an adequate picture of the mitochondrial unit, the fact of an organized internal structure seems to be by far the most important one. Given this organization the question of whether there is a membrane or not becomes of lesser importance. However, for an understanding of the form in which coenzymes like the pyridine nucleotides exist in the mitochondrion, whether free or combined (39, 40, 41), a decision on the membrane question might be decisive.

[5] See also under "Electron Transport System:" DPNH oxidase.

[6] Internal structures which are visible in some electron micrographs have been interpreted in terms of small tubules (38).

Many investigators have described the swelling of mitochondria exposed to hypotonic salt solutions (41, 42, 43). This process of water imbibition can soon lead to irreversible loss of the capacity for oxidative phosphorylation. ATP[2] or active metabolism not only prevents this swelling phenomenon from taking place but also can reverse the process once it has taken place (44, 45). These experiments on the osmotic behavior of mitochondria have assumed great importance in that they constitute the principal line of evidence in favor of the view that there is an active membrane enveloping the mitochondrion which regulates the inflow of solutes and water (46, 47). Osmotic changes *per se* are, however, not necessarily diagnostic of a system containing a membrane. Gelatin or myosin for example also respond osmotically in solutions of varying ionic strength. While osmotic changes may be consistent with the postulation of an active membrane they do not provide any proof on that score.

Bartley & Davies (48) have conducted a very significant study of ion transport by kidney mitochondria. They have shown that actively metabolizing mitochondria can concentrate sodium, potassium, hydrogen, and phosphate ions to a small but significant extent. Macfarlane & Spencer (49) previously had made similar observations on liver mitochondria. The interpretation of the development of this ion concentration gradient during active metabolism is still difficult. According to Bartley & Davies

> there remains the possibility that the mitochondria behave like a more complex Donnan system with two immobile electrolytes of opposite charge such as mixtures of cation and anion exchange resins which can maintain high gradients of both cations and anions in salt solution. In this case metabolic energy might be needed to generate or maintain the immobile charge centers. The actual steady-state exchange could then take place without further energy supplies.

Actively metabolizing kidney mitochondria can also concentrate α-ketoglutarate, fumarate, and pyruvate (48). Whether this is related to the amounts of these substrates which are bound to their respective enzymes is still an open question.

When mitochondria are freshly prepared with adequate precautions adenosinetriphosphatase activity is very low (50). On storage or treatment with 2,4-dinitrophenol, adenosinetriphosphatase activity becomes manifest, and coincident with this unmasking of activity the capacity for oxidative phosphorylation declines rapidly. This phenomenon which has been thoroughly studied by Potter *et al.* (51) and by Lardy & Wellman (52) points up the complexity of the problem of maintaining mitochondria in a fully active state. Any inadequacy in the execution of the mitochondrial preparation is reflected in the emergence of adenosinetriphosphatase activity and the concomitant loss of integrated enzymatic reactions. An interesting new development is the observation by Harman & Kitiyakara (53) that under appropriate conditions this emergence phenomenon is reversible. Thus mitochondria exposed to high salt concentrations have high adenosinetriphosphatase activity. When these mitochondria are placed in an isotonic medium

adenosinetriphosphatase activity declines again to a low rate. Polis & Shmukler (54) have described the isolation from liver mitochondria of a hemoprotein in homogeneous state which they named mitochrome I and which induces adenosinetriphosphatase activity when added to freshly prepared mitochondria. The authors conclude that a labile mitochrome-phosphate complex is formed by transphosphorylation with ATP and that the decomposition of this complex liberates inorganic phosphate.

As efforts have been directed towards preparation of mitochondria from different sources, new tactics, and reagents for stabilizing enzymatic activity have been devised. Slater & Cleland (55) have found versene to be effective in the stabilization of skeletal muscle mitochondria (sarcosomes); likewise versene and plasma albumin were found to stabilize the activity of insect sarcosomes (56, 57, 58). The instability observed in the preparation of some mitochondrial suspensions probably stems from the action of extramitochondrial enzymes and reagents and may not reflect any fundamental difference in properties between mitochondria from different cells. Millerd & Bonner (21), Stafford (59), and McClendon (60) have made notable contributions to our knowledge of the special conditions and requirements for the preparation of mitochondria from plant sources. Similarly, Nossal (61, 62) has worked out techniques for the study of yeast mitochondrial preparations which appear to be very unstable.

Clearly enormous progress has been made in our understanding of the mitochondrial system as compared to the position in 1947 when the concept of the integrated complex of enzymes, the cyclophorase system, was first proposed (63). The area of uncertainty is now one of defining both the nature and extent of mitochondrial organization. There is no reason to believe that the full extent of mitochondrial reaction sequences has been already discovered. Until more is known of the locale of major synthetic processes which have yet to be reconstructed *in vitro* the possibility of mitochondrial participation in these processes cannot be excluded.

Electron Transport System

Until very recently concepts of the mitochondrial electron transport system of aerobic cells were based almost exclusively on the early qualitative spectroscopic observations of Keilin and the later quantitative spectrophotometric studies of Chance. The spectroscopic data were obtained largely by the examination of whole cells, homogenates, and crude cell particulates. Previous reviews have dealt extensively with the classical version of the electron transport system and with the interpretations of the spectroscopic examination of unfractioned systems (64 to 67). In particular the unique contributions of the laboratory of Chance to the whole area of electron transport from pyridine nucleotides to the cytochrome system have been recently discussed (68, 69). The present review will focus attention primarily on recent efforts to deduce the nature and properties of the electron transport system from the direct study of the isolated components.

A variety of methods have been described for the separation and purification of the particulate elements which collectively make up the electron transport system. Eichel *et al.* (70) and Dannenberg & Kiese (71) have used desoxycholate as a reagent for separating cytochrome oxidase and cytochrome-*b*. Smith & Stotz (72) have isolated cytochrome oxidase from heart muscle first by digesting a crude particulate suspension of the enzyme with trypsin in presence of cholate and then by fractionating the clarified cholate extract with ammonium sulfate. Strittmatter & Ball (73) have applied the technique of fractional ultracentrifugation to the isolation of a particulate hemoprotein from rat liver which shows activity as a DPNH dehydrogenase. Kun (74) extracted a protein from rat liver particles by treatment with isobutanol and carboxymethylcellulose, which showed the spectrum of cytochrome-*b* and activity as a DPNH and TPNH cytochrome-*c* reductase. While these studies are not without some ambiguity of interpretation nonetheless they do provide evidence that certain entities of the original particulate material are separable, that these entities are well-defined, and that the study of the electron transport mechanism can be approached by way of the direct examination of the isolated components.

Succinic dehydrogenase.—During the past year the isolation of succinic dehydrogenase in various forms was announced by several laboratories. Stotz and collaborators (75 to 78) obtained a preparation by their cholate technique which was 20 to 30 times more active than the original heart homogenate and was free from the one or more components which are believed to link succinic dehydrogenase to cytochrome-*c*.[7] Succinic dehydrogenase has also been isolated in two forms by procedures not involving preliminary treatment of the particulate starting material with bile salts, as a particle by Green *et al.* (31, 80) and as a soluble protein of relatively low molecular weight by Singer & Kearney (30). The interrelationships of these two forms may provide an important clue to an understanding of the organization of the complex enzymes involved in electron transport. For ease of presentation the particulate form of the dehydrogenase will be referred to in this review as the succinic dehydrogenase complex (SDC) while the soluble form will be referred to as the succinic dehydrogenase (SD). According to the available evidence SDC may be conceived of as a precise, reproducible enzymatic unit made up of a number of components linked together by strong bonds and no different in respect to uniformity than any other complex enzyme system such as milk xanthine oxidase (81), α-ketoglutaric dehydrogenase (82), or yeast lactic dehydrogenase (83). SDC can be cleaved under appropriate conditions into SD and some residue as yet uncharacterized. Both SDC and SD carry out the same primary reaction, viz., the reduction of the primary prosthetic group by succinate. But that is the extent of the similarity. SDC has an elaborate electron conductor system or network for passage of electrons from the primary prosthetic group to external electron acceptors. SD is lacking in all of the components of this conductor system.

[7] This work has been more extensively discussed by Anfinsen & Kielley (79).

SDC has been separated from other particulate enzymes and from soluble enzymes by extraction from frozen and thawed mitochondria with 10 per cent tertiary amyl alcohol and by fractional centrifugation in the Spinco ultracentrifuge. The dehydrogenase activity of the best preparations is 15 to 20 times greater than that of the original mitochondria. If one assumes on the basis of inhibition experiments that one mole of antimycin combines with one mole of SDC then the molecular weight of such a unit would be 1.4×10^6, and per unit there would be five heme groups[8] and about 20 atoms of nonporphyrin iron. SDC contains 60 per cent by weight of lipide. The calculated molecular weight cited above is on a lipide-free basis.

SDC can react with a variety of electron acceptors such as phenazine methosulfate, ferricyanide, indophenol, and cytochrome *c* but not with molecular oxygen. Antimycin A inhibits the oxidation of succinate only when cytochrome *c* acts as electron acceptor. The phenazine reducing activity of SDC is more stable than the cytochrome reducing activity during storage and manipulation of the enzyme.

When SDC is treated with trypsin according to the procedure of Smith & Stotz (72), several types of soluble succinic dehydrogenases are released, one form of which is similar to the Singer-Kearney SD, while the others are more complex and contain one or more heme groups per molecule of enzyme [Green, D. E., and Mii, S. (Unpublished data)]. A particulate residue remains which contains no heme but which contains a large proportion of the nonporphyrin iron.

The Singer-Kearney SD catalyzes the oxidation of succinate by phenazine methosulfate and to a small extent by ferricyanide; it is inactive with indophenol or cytochrome-*c*. At the highest purity level the enzyme is 140 times more active per mg. dry weight than is the original mitochondrial suspension. The highly purified enzyme has an amber brown color and contains flavin but contains no heme groups. The brown color of the enzyme is partially bleached by succinate. Malonate in sufficient concentration prevents this reduction. There is about 1 gm. atom of iron in 200,000 gm. of enzyme protein. The iron is released from combination with the protein in 5 per cent trichloracetic acid solution. The enzyme has an absolute requirement for inorganic phosphate [Singer, T. P. (Unpublished data)].

In the light of these findings one may now inquire what place in the total picture can be assigned to (*a*) cytochrome-*b*, (*b*) Slater's factor, and (*c*) cytochrome-*c* linking factor. If the concept of SDC developed above is a valid one then the designation of cytochrome-*b* (defined as the cytochrome corresponding to a three banded spectrum with peaks at 430, 530, and 564 mμ) refers to the constituent heme groups of SDC. These heme groups of SDC can be looked upon as being no different in character than the heme groups of hemoglobin or of yeast lactic dehydrogenase (83). With respect to

[8] The term heme groups will be used in this review to designate iron-porphyrin containing groups in general, irrespective of the valency state of the iron, the nature of side chains and the presence of additional groups linked to the iron.

the Slater factor and the cytochrome-*c* linking factor, these refer to groupings in SDC which are involved in the reaction with cytochrome-*c*, and there is no evidence as yet that these components are still functional when detached from SDC[9]

DPNH oxidase.—A particulate enzyme unit has been extracted and purified from beef heart mitochondria by Green *et al.* (84) which catalyzes the oxidation of DPNH by molecular oxygen but which catalyzes at a much lower rate only the reduction of cytochrome-*c* by DPNH or the oxidation of reduced cytochrome-*c* by molecular oxygen. When this purified particulate system is treated with desoxycholate the capacity to catalyze the over-all oxidation of DPNH by molecular oxygen is greatly reduced whereas the capacity to catalyze the oxidation of DPNH by cytochrome-*c* or t he oxidation of reduced cytochrome-*c* by molecular oxygen increases about 25-fold. The curve describing the loss of DPNH oxidase activity is the mirror image of the curves describing the emergence of DPNH cytochrome-*c* reductase activity and of reduced cytochrome-*c* oxidase activity. These observations have been interpreted as follows. DPNH oxidase exists as an enzymatic unit in which electrons are transferred from the primary prosthetic group to the final electron acceptor (molecular oxygen) by way of a series of intermediary electron carriers such as heme groups and metals. This unit has the character of an impenetrable "closed" unit in the sense that electrons can only enter by way of DPNH and leave by way of oxygen. There is thus little possibility of interaction with electron acceptors like cytochrome-*c* or dyes. When the unit is exposed to desoxycholate the conducting circuit is interrupted in the sense that a barrier is raised to the flow of electrons at some point, possibly by the loss of some component. Such an "opened" DPNH oxidase unit can now interact with acceptors like cytochrome-*c*, probably by thermal collisions, which then undergoes a cycle of reduction by DPNH in the reducing moiety of the unit and of oxidation by molecular oxygen in the oxidizing moiety. In other words the "opened" DPNH oxidase

[9] The observations of Stotz and collaborators on the components of the succinic dehydrogenase which were described last year (79) may at first glance appear to be inconsistent with the picture presented above. However, the differences are those of terminology and emphasis rather than of fact. These authors (75) have obtained a preparation, by the use of cholate, which catalyzes the oxidation of succinate by cytochrome-*c*. This is called a preparation of "SC factor," "linking succinic dehydrogenase with cytochrome-*c*." In the terminology used in this review, this would correspond to particulate SDC. It has so far not been reported that the link with cytochrome-*c* can be separated in an active form from the primary dehydrogenase. It seems possible that soluble succinic dehydrogenase, which no longer interacts with cytochrome-*c*, could regain the capacity for this interaction, when supplemented with some fragments of SDC. However, in view of the findings reported in the following section on DPNH oxidase, the significance of such an interaction with added, previously isolated cytochrome-*c* remains to be determined. The entity, which Stotz and collaborators refer to as succinic dehydrogenase, obtained by cholate solubilization, is probably similar to the soluble fragments obtained from SDC by trypsin digestion.

now behaves as if it were a complex of two enzymes, DPNH cytochrome-*c* reductase and cytochrome oxidase which are no longer in electronic communication with one another. According to this interpretation both DPNH cytochrome-*c* reductase and reduced cytochrome oxidase activities are, in this case at least, artifactual in the sense that cytochrome-*c* is not the normal acceptor for electrons emerging from DPNH, and reduced cytochrome-*c* is not the normal donor of the electrons which are ultimately transferred to molecular oxygen. The opening of the DPNH oxidase unit has, so to speak, created an artificial situation in which cytochrome-*c* is closing the opened circuit again, perhaps by taking the place of some component which normally provides the link between the two moieties of the conductor system. It should be pointed out that there is no cytochrome-*c* present in the DPNH oxidase preparation and that cytochrome-*c* is not liberated in consequence of the treatment with desoxycholate.

The ease with which DPNH oxidase can be opened by reagents other than desoxycholate, e.g., detergents and alcohols, imposes restrictions on the scope of procedures which can be used for further purification of the "closed" particulate unit. A preparation can, however, be obtained (85) which is very low in succinic and α-ketoglutaric dehydrogenases and free of the other enzymes of the citric acid cycle but which in the terminology used above can be considered to be only partially closed (about 50 per cent). This preparation shows two types of heme groups, a red one with the spectral characteristics of the heme groups of SDC (bands with maxima at 525 and 555 mμ) and a green one with one band at 607 mμ. The amounts of these two heme types are roughly equal. The Soret band is a broad composite of the Soret bands of the two heme types. When this preparation is treated with trypsin in presence of cholate, separation of the two heme types and of the catalytic activities can be achieved by ammonium sulfate fractionation [Mackler, B. (Unpublished data)]. An easily sedimentable fraction contains the green heme group and copper (two atoms of copper per heme group) while a difficultly sedimentable particulate fraction contains the red heme group and iron (five atoms of total iron per heme group). The former shows cytochrome-*c* oxidase activity while the latter shows DPNH dehydrogenase activity.[10] Thus the green heme group and copper (some nonporphyrin iron as well) can be assigned to the cytochrome-*c* oxidase moiety while the red heme group and the bulk of the nonporphyrin iron can be assigned to the DPNH dehydrogenating moiety of the complex DPNH oxidase. Mackler *et al.* (85) have shown that this moiety which has DPNH dehydrogenase activity and which contains a heme group also contains flavin in the ratio of one molecule per five heme groups.

A DPNH cytochrome-*c* reductase of high purity had been isolated previously by Mahler *et al.* (86) and later shown to be a ferroflavoprotein by Mahler & Elowe (87). Each molecule of this enzyme contains four atoms of

[10] With dyes as electron acceptors; cytochrome-c reductase activity has been lost during the treatment necessary for separation.

iron per molecule of flavin. No heme group is present in this enzyme.

There is thus evidence that DPNH dehydrogenase exists in at least three different forms: (*a*) as the closed complex, "DPNH oxidase," which reacts with molecular oxygen, (*b*) as the form which contains a heme group and which is obtained by tryptic digestion of the former enzyme, and (*c*) as the soluble ferroflavoprotein of Mahler & Elowe. Finally the last named can be degraded to a simple flavoprotein without iron (87) which is similar to Straub's diaphorase. This evidence of the change in state of an enzyme seems very impressive.

One point in particular should be stressed in the context of this discussion. The transport of electrons from DPNH to the final acceptor does not follow the same pathways in the three different forms of the DPNH oxidizing enzyme. The Mahler-Elowe enzyme probably reacts by way of the iron atoms chelated with the flavin; the heme-containing antimycin-insensitive enzyme reacts by way of a pathway which presumably involves the heme group; finally DPNH oxidase in the closed state has no possibility of reacting with the same acceptors as the other two enzymes except after treatment with desoxycholate. The reactions catalyzed by the "closed" or "opened" DPNH oxidase are sensitive to antimycin. Again it must be pointed out that the interaction with cytochrome-*c* may have no physiological meaning. Even though cytochrome-*c* is of natural origin, nonetheless the reaction in question may still be artifactual.

Various investigators have described the isolation of a particulate fraction containing a green hemoprotein which catalyzes the oxidation of reduced cytochrome-*c* by molecular oxygen. The laboratories of Wainio and Kiese have used relatively high concentrations of desoxycholate (about 2 per cent) to separate reduced cytochrome-*c* oxidase from DPNH dehydrogenase (70, 71). Smith & Stotz (72) have introduced the technique of tryptic digestion as a device for accomplishing the separation. Mackler has used the same technique but has applied it to the purified DPNH oxidase mentioned above and thus has been able to recognize two products of the digestion process [Machler, B. (Unpublished data)].

The meaning of terms such as "solubilization" of particulate enzymes is ambiguous and requires some clarification. Desoxycholate or cholate "solutions" of succinic dehydrogenase and cytochrome oxidase can be transparent and without evidence of any Tyndall effect. These "solutions" which should not be confused with true solutions become turbid as soon as desoxycholate or cholate is dialyzed out and the original particulate character of the "solubilized" enzyme is restored.

For certain approaches to the study of a particulate unit the physical state of the enzyme must be accepted and appropriate methods devised for separating this particle from other extraneous materials. On the other hand a portion of a particulate unit may be detached in soluble form as in the case of the succinic dehydrogenase but again this is not solubilization but degradation in the same sense that glucose is a degradation product of starch

and not a solubilized version of starch. Mahler & Elowe's DPNH cytochrome-*c* reductase is not a solubilized version of the original particulate DPNH oxidase. It is a derivative, soluble fragment of the original unit which lacks many of the properties and characteristics of the original unit.

Inherent in the classical picture of the electron transport system is the notion that there are two cytochromes both with an absorption band at about 605 mμ which differ only in their capacity to react with carbon monoxide and cyanide. These have been referred to as *a* and a_3 respectively, only the latter being assigned the role of reacting with carbon monoxide and cyanide. Examination of purified preparations of cytochrome oxidase has failed to disclose any evidence for the separation of two green heme groups with bands at 605 mμ (72).[11] It would be desirable to have more definite support available for the view that a separate entity intervenes in the electron transport pathway between cytochrome-a_3 and cytochrome-*c* in animal tissues. Neither the hypothetical cytochrome *a* nor cytochrome-*c* appear to be implicated in the DPNH oxidase system.

Other components of the cytochrome system.—In addition to the studies described above in which the interrelationships of the components of the terminal electron system are deduced from the direct study of the enzyme units a great resurgence of interest in the identification of new cytochromes has developed. These cytochromes may be divided into two groups, one which includes hemoproteins similar to but not identical with cytochrome-*c* and the other which includes cytochromes with spectral properties similar to those of cytochrome-*b*. There may well be a fundamental difference between these two types of cytochromes. The members of the cytochrome-*c* group are capable of acting as electron acceptors for appropriate dehydrogenase systems whereas the cytochromes of the *b* group are inert in that respect. Elsden, Kamen & Vernon (88) have isolated a cytochrome-c_2 from *Rhodospirillum rubrum* whose absorption bands are identical with those of mammalian cytochrome-*c*. The reduced hemoprotein is not autoxidizable in air, nor is it oxidized by cytochrome oxidase of heart muscle. The oxidized form is reducible by DPNH cytochrome reductase of animal tissues. Egami *et al.* (89) have extracted from cells of halotolerant bacteria a soluble cytochrome-b_4 whose absorption bands are similar to those of SDC. Pappenheimer & Williams (90) correlated DPNH oxidase activity in cecropia silkworms with the presence of a cytochrome which they refer to as cytochrome-b_5. Chance & Williams (91) studied a similar cytochrome in rat liver microsomes. Hübscher, Kiese & Nicolas (92) have separated fractions from beef heart particles which answer to the description of cytochrome-*b*. No enzymatic activity other than that of succinic dehydrogenase has been reported for these fractions. Appelby & Morton (83) were able to crystallize the so-called cytochrome-b_2 from yeast which appears to have a relationship to lactic dehydrogenase similar to that of cytochrome-*b* to succinic dehydrogenase, i.e., cytochrome-b_2 is part of the hemoflavoprotein, yeast lactic dehydro-

[11] Mackler, B. (unpublished data).

genase. Tissieres *et al.* (93, 94) have studied the cytochrome system of various strains of *Neurospora*. Smith (95) has made a thorough study of yeast and bacterial cytochromes. This work is discussed in a recent review by Smith (96) in which she has ably summarized the present state of our knowledge of bacterial cytochromes. In the plant field, Hill (97) has described the properties of a cytochrome-*b* component isolated from chloroplasts while Lundegårdh (98) reports spectroscopic evidence of a cytochrome-*dh* in wheat roots which is similar to but not identical with cytochrome-*b*. Other work in the plant field has centered around the demonstration that the cytochrome system mediates the terminal electron transport in higher plants (99 to 102). The participation of pigments of the cytochrome class in light-induced reactions is indicated by the studies of Duysens (103), Vernon & Kamen (104), and of Lundegårdh (105).

Metalloflavoproteins

During the past year the concept of metalloflavoproteins has become firmly established, a concept which appears to have far reaching implications for the problem of electron transport (106). Three independent lines of evidence have led to this development. Mahler (107) discovered the presence of copper in the green fatty acyl CoA^3 dehydrogenase of beef liver. Nicholas *et al.* (108, 109) found that *Neurospora* grown in absence of molybdenum became deficient in nitrate reductase while DeRenzo *et al.* (110) and Richert & Westerfeld (111) implicated molybdenum as the active principle of a liver supplement which stimulated production of intestinal xanthine oxidase in rats maintained on a restricted diet. Thus far six metalloflavoproteins have been described in the literature and three chelating metals have been implicated, viz., copper, iron, and molybdenum. Xanthine oxidase of milk (81, 112 to 115), aldehyde oxidase of liver (116), hydrogenase of *Clostridium pasteurianum* (117), and DPNH nitrate reductase of *Neurospora* (108, 109) are molybdoflavoproteins; DPNH cytochrome-*c* reductase of heart (87) is a ferroflavoprotein, and the green acyl CoA dehydrogenase is the only recorded cuproflavoprotein (107). The reduction of nitrite to ammonia by DPNH, which extracts of *Neurospora* catalyze, proceeds according to Nason *et al.* (118) in at least two steps for both of which addition of metal and flavin are required. Thus further additions to the metalloflavoprotein list are indicated.

In all cases which have been studied so far it has been established that the metal facilitates the interaction of the reduced flavoprotein with one-electron acceptors such as cytochrome-*c* and ferricyanide. The interaction with two-electron acceptors such as organic oxidation-reduction dyes does not require the presence of metal. The metal requirement in the former instance has pointed up a kinetic problem, viz., that the stepwise removal of electrons from a two-electron donor to a one-electron acceptor is an improbable reaction in absence of an appropriate catalyst. In a metalloflavoprotein chelate system the metal probably can bind at least two molecules

of the electron acceptor and the shift of electrons then becomes intramolecular rather than intermolecular. Metalloflavoproteins would then in effect facilitate a ternary collision by combining reductant and two molecules of oxidant in the same chelate system.

At first glance the requirement of molybdenum for nitrate reduction may appear to be out of line with the role of metal in catalyzing interactions with one-electron acceptors. There are two factors which have to be considered in this context. Nitrate is not an oxidizing agent in the same sense that oxidation-reduction dyes, ferricyanide, and cytochrome-*c* are considered to be oxidizing agents. Metal chelation might therefore be an essential preliminary to the activation of nitrate as an electron acceptor. Secondly, the interaction of reduced flavins with oxygen and oxidation-reduction dyes involves a transfer of hydrogen atoms whereas the molybdenum-catalyzed reaction with one-electron acceptors involves only a transfer of electrons. Nitrate reduction would of course fit into the category of reductions which proceed by electron-transfer exclusively.

The molybdenum catalyzed interaction of xanthine oxidase, hydrogenase, and aldehyde oxidase with one-electron acceptors has been shown to have an absolute requirement for inorganic phosphate ions. The full significance of this requirement is not clear. If the chelating metal is reducible in the form only of its phosphate adduct and not of its aquo counterpart then the possibility exists that coincident with reduction of the metal a covalent bond between phosphate and the metal might be formed. Since such a compound would be stable only in the reduced state, the bound phosphate would be lost again on reoxidation. This might account for the fact that the oxidation reaction goes on indefinitely in absence of any acceptor system for the phosphoryl group. The phosphate requirement does not apply to the nonmetal requiring interaction of the flavoprotein enzyme with oxidation-reduction dyes.

The proportions of metal to flavin are quite variable among the different metalloflavoproteins. In DPNH cytochrome-*c* reductase there are four atoms of iron per molecule of flavin; in the green acyl CoA dehydrogenase the copper: flavin ratio is 2 while the molybdenum: flavin ratio in xanthine oxidase is 0.5. There is a possibility that the last ratio is lower in the isolated enzyme than in the original enzyme. Molybdenum is more readily removed from combination with its respective apoenzyme than either iron or copper during purification procedures such as fractionation with ammonium sulfate or dialysis.

Liver aldehyde oxidase appears to be an example of a metalloflavoprotein containing a heme group (protoheme). This is reminiscent of DPNH cytochrome-*c* reductase of beef heart which as mentioned in the preceding section has been isolated both as a ferroflavoprotein and as a ferroflavoprotein containing a heme group. The characterization by Appleby & Morton (83) of crystalline yeast lactic dehydrogenase as a flavoprotein linked to a heme group suggests a third example of this class since the enzyme in question

can react with cytochrome-*c* and consequently is likely to contain some as yet uncharacterized metal chelated to the flavin.

Milk xanthine oxidase contains a chromophoric group other than flavin which accounts for the deep red color of concentrates of the enzyme. Richert & Westerfeld (115) have found considerable amounts of iron in the enzyme which they suggest might account for some of the anomalies in the spectrum of the flavoprotein enzyme.

Mackler *et al.* (81) have confirmed the disputed (119) conclusion of Corran *et al.* (120) that milk xanthine oxidase is associated with DPNH dehydrogenase activity. At the highest purity level of xanthine oxidase the ratio of hypoxanthine to DPNH dehydrogenase activity is the same as in the original starting material. Molybdenum is required for the interaction of both hypoxanthine and DPNH with one-electron acceptors. Furthermore a direct interaction between hypoxanthine and DPN without addition of a carrier is demonstrable, a reaction which renders unlikely the possibility that the two activities reside on separate enzymatic units.

The hitherto puzzling feature of milk xanthine oxidase that the prosthetic flavin is reduced only partially in presence of hypoxanthine and not at all in presence of DPNH can be explained in terms of the low potential of the metalloenzyme (81, 106).

Avis *et al.* (121) have prepared milk xanthine oxidase in crystalline form for the first time. The catalytic activity and the purity of the crystalline preparations as judged by the flavin to protein ratio are not greatly different from those reported by Horecker & Heppel (122), Mackler *et al.* (81), and Morell (119).

Only in the case of DPNH cytochrome-*c* reductase (87) has it been possible to show directly that the chelating metal is under going valency changes during the interaction with one-electron acceptors. Iron lends itself to such a demonstration since convenient methods are available for distinguishing between the two valency states of the metal and since the removal of the metal from the protein under mild conditions (5 per cent trichloracetic acid) can be readily effected in this case. The reversible reduction and oxidation of copper in the fatty acyl CoA dehydrogenase (107) has been inferred from the fact that the band at 680 mμ, which undoubtedly is contributed by the copper moiety in the cupric state, is abolished when substrate is added to the enzyme and is restored when the reduced enzyme is reoxidized in air. Nicholas & Nason (122a) were able to show that chemically reduced molybdenum can serve as an electron donor for nitrate reduction in presence of the enzyme and in the absence of added TPNH.

Mahler *et al.* (123) have compared the rate of reaction of metal-containing and metal-free flavoproteins with a series of quinones of different oxidation-reduction potentials. From the decisive way in which the presence of the metal alters the pattern of this interaction they have concluded that the metalloflavoprotein forms one single resonating system and is not merely a compound of flavin and some metal.

A variety of oxidizing enzymes, flavoproteins included, are known to interact with quinones of appropriate oxidation-reduction potentials. In view of this lack of specificity for quinones as oxidizing agents there is some difficulty in assessing the significance of various "quinone reductases" which have been reported recently (124 to 128).

Electron Transferring Flavoprotein

The metalloflavoprotein is not the only enzymatic device for facilitating the interaction of two-electron donors with a one-electron acceptor. Crane & Beinert (129) have recognized the operation of a new principle in the interaction of the two fatty acyl CoA dehydrogenases (130, 131) with various electron acceptors. Both of these flavoprotein enzymes can be purified to a point where the oxidized forms are reducible by their appropriate substrates but the reduced forms lack the capacity to interact with oxygen, indophenol, or cytochrome-*c*. On addition of another flavoprotein all these interactions take place. According to the convention in use among enzyme chemists this flavoprotein would have to be called "reduced acyl CoA dehydrogenase-cytochrome *c* reductase." Since a more convenient term is desirable we have elected to call this flavoprotein the electron transferring flavoprotein (ETF), to point up its most significant feature. ETF undergoes a cycle of reduction by the reduced fatty acyl CoA dehydrogenases and then of oxidation by any of the various electron acceptors. The fatty acyl CoA dehydrogenases represent the first cases of flavoproteins which in their reduced forms can be oxidized only by another specific flavoprotein. Thus two flavoproteins in series are needed to transfer electrons from substrate to final acceptor. The question is raised by these findings whether the occurrence of a primary dehydrogenase together with a unit like ETF may not be a more general phenomenon in flavoprotein systems.

When freshly prepared, ETF is active with a variety of electron acceptors. On storage the capacity for interaction with cytochrome-*c* declines and eventually disappears, whereas the capacity for interaction with other one- and two-electron acceptors is unimpaired. The available evidence appears to exclude the possibility that ETF is a metalloflavoprotein. As yet the mechanism of action of ETF is unknown.

At the time of the earlier work of Green *et al.* (130) and Mahler (107) on the green butyryl CoA dehydrogenase, it was not known that an additional flavoprotein, viz. ETF, intervenes in the catalysis ascribed to the fatty acyl CoA dehydrogenases. The discovery of ETF obviously necessitates a re-evaluation of some of the earlier observations, specifically of those which deal with the role of copper in butyryl CoA dehydrogenase.

Oxidative Phosphorylation

The knowledge that the aerobic oxidation of certain metabolites is linked to esterification of inorganic phosphate has been available for 15

years.[12] This phenomenon of oxidative phosphorylation which has been localized unambiguously in mitochondria is characteristic of only two over-all oxidative processes both involving molecular oxygen: (*a*) the oxidation of intramitochondrial DPNH to DPN, and (*b*) the oxidation of succinate to fumarate.[13] The oxidation of α-ketoglutarate by DPN[2] is also linked with phosphate esterification, but since this process does not involve the terminal electron transfer system it is usually placed in a separate category. There are probably three molecules of phosphate esterified per atom of oxygen required for oxidation of mitochondrial DPNH and two for the oxidation of succinate (9, 11). The link between the oxidation of DPNH or succinate and phosphorylation is abolished by DNP[2] without the rate of oxidation being affected except by way of stimulation. Damage to the mitochondrion has the same end effect as addition of DNP, viz., loss of capacity for oxidative phosphorylation. The extraordinary fragility of the aerobic phosphorylation system is at once the marvel and despair of those who have elected to study the phenomenon. Oxidative phosphorylation has been reproduced in a nonmitochondrial system of pig heart muscle (135), but this system still does not lend itself to systematic analysis. The barrier to a clear understanding of oxidative phosphorylation is and has been our ignorance of the details of the electron transport mechanism. Now that progress is being made in that direction we may anticipate parallel developments in the analysis and reconstruction of oxidative phosphorylation.

During the past year Sanadi, Gibson & Ayengar (136) have reconstructed in a system of soluble enzymes from animal tissues the steps involved in the phosphate esterification which accompanies the oxidation of α-ketoglutarate by DPN, (see equations 1 to 3 below):

$$\alpha\text{-Ketoglutarate} + DPN^+ + CoA \rightarrow \text{Succinyl CoA} + DPNH + H^+ + CO_2 \quad 1.$$

$$\text{Succinyl CoA} + P_i + GDP(IDP) \rightleftharpoons \text{Succinate} + GTP(ITP) + CoA \quad 2.$$

$$GTP(ITP) + ADP \rightleftharpoons GDP(IDP) + ATP. \quad 3.$$

Reaction 1 is catalyzed by the soluble α-ketoglutaric dehydrogenase of pig heart muscle; reaction 2 by the phosphorylating enzyme from pig kidney or heart muscle; and reaction 3 by a nucleoside diphosphokinase (137, 138) type of enzyme prepared from beef or pig heart. GDP or IDP[2] but no other of the known diphosphonucleotides can serve as phosphate acceptor in reaction 2. However, by virtue of transphosphorylations GTP or ITP[2] can react with many other nucleotides.

By now the mono-, di-, and triphosphoderivatives of practically every ribonucleotide and of some desoxyribonucleotides have been shown to exist in free form in tissue extracts (139 to 144). In addition varied types of internucleotide transphosphorylations have been discovered (137, 138, 145, 146,

[12] Cf. Hunter (132) for an excellent review on oxidative phosphorylation covering the developments up to 1951.

[13] The oxidation by liver mitochondria of choline (133) and of sulfite (134) is also accompanied by phosphate esterification.

147). The student of phosphorylation phenomena will have to take these transphosphorylation reactions into account before any valid decision can be reached with respect to which nucleotide in which phosphorylated form acts as the primary phosphate acceptor. Although it has been shown (136) that GTP is the primary acceptor in the substrate level phosphorylation accompanying α-ketoglutarate oxidation, evidence from Potter's laboratory (148) "suggests that the adenine nucleotides are obligatory or preferential intermediates" in many oxidative phosphorylation reactions.

In further work on the phosphorylating enzyme Kaufman & Alivisatos (149) have obtained from spinach a highly purified preparation which catalyzes the following phosphorylation reaction:

$$\text{Succinyl CoA} + P_i + \text{ADP} \rightleftarrows \text{Succinate} + \text{CoA} + \text{ATP} \qquad 4.$$

GDP is not required for the phosphorylation of ADP though the possible presence of bound DGP in the enzyme has not been excluded. Exchange studies with P^{32} have led Kaufman & Alivisatos to the hypothesis that enzyme bound phosphoryl CoA is probably the intermediate in reaction 4.

According to Sanadi *et al.* (150) arsenate catalyzes the deacylation of succinyl CoA to succinate and CoA in absence of GDP. Furthermore GTP in presence of arsenate is converted to GDP and inorganic phosphate essentially by a reversal of reaction 2. These results on arsenolysis suggest that reaction 2 is composite in nature and may consist of at least two consecutive reactions.

The use of inorganic phosphate labeled with O^{18} and P^{32} has led to the recognition by Boyer *et al.* (151) of some new facets of oxidative phosphorylation in a mitochondrial system. According to these authors the exchange of the oxygen of inorganic phosphate labeled with O^{18} with that of water, which was first observed by Cohn (152), can go on independently of oxygen uptake and of net change in the concentration of inorganic phosphate and ATP. Similarly there is exchange of P^{32} between labeled inorganic phosphate and ATP even in absence of measurable mitochondrial oxidation. This exchange of phosphate is less rapid than the exchange of oxygen. Dinitrophenol and anaerobiosis respectively abolish and inhibit the exchange phenomena. Control experiments rule out the possibility that nonoxidative transfer reactions can account for the extent of exchange (151, 153). Boyer *et al.* conclude that in the process by which inorganic phosphate becomes esterified during terminal oxidation there is a loss of oxygen from inorganic phosphate and that reversal of this process would lead to exchange of the oxygens of inorganic phosphate with water as seen from the over-all equation:

$$\text{ADP} + P_i^{32} \rightleftarrows \text{ATP} + H_2O \qquad 5.$$

According to this interpretation reversibility of the primary oxidative event leads to exchange of oxygen between water and the phosphate molecule which is taken up. The phosphoryl derivative formed can then exchange with phosphate in ATP but never to a greater extent than the primary and preceding exchange of oxygen.

Boyer *et al.* invoke Taube's (154) conception of a bridge atom and apply it to oxidative phosphorylation. The interaction of two components of the electron transfer system, e.g., of a metalloflavoprotein with cytochrome-*c*, may be bridged through the oxygen of a phosphate molecule. Coincident with electron transfer the bridge phosphate becomes linked with one or the other component in the form of an active phosphoryl derivative capable of interacting with ADP to form ATP. Slater (155) proposed in a recent review a mechanism of oxidative phosphorylation during electron transport which is based essentially on analogy with reaction schemes describing substrate level phosphorylations (149, 150, 156). Supporting experimental evidence for these stimulating speculations is not yet available.

The laboratories of both Lehninger (157, 158) and Lardy (159) have provided new data bearing on oxidative phosphorylation accompanying the mitochondrial oxidations respectively of reduced cytochrome-*c* and ascorbic acid. The P/O ratio approaches 1 in both cases. These results show that under certain conditions appropriate reducing agents can plug in as it were into the electron transport chain and their oxidation by molecular oxygen can then lead to limited oxidative phosphorylation.

There has been a renewed interest in the question of the maximum value for the P/O ratio obtained during oxidation of α-ketoglutarate. Slater & Holton (160) have questioned the validity of reported values approaching 4. However, the basis of their criticism has been challenged by several investigators (157, 159, 161, 162).

Martius & Nitz-Litzow (163) found that the P/O ratio for the oxidation of β-hydroxybutyrate by liver mitochondria of vitamin K deficient chicks is significantly and consistently lower in value than that for mitochondria of normal animals. In addition 10^{-5} *M* vitamin K added *in vitro* (but not 2-methyl-1,4-naphthoquinone) raises the P/O ratios of the mitochondria from deficient animals to about the normal levels.

Mudge and collaborators (164, 165) have been studying systematically the formation of phosphoenolpyruvate by liver mitochondria acting upon α-ketoglutarate in presence of inorganic phosphate and high concentrations of DNP but in absence of added adenine nucleotides. The recent discovery by Utter & Kurahashi (166) of a system which catalyzes the reaction,[14]

$$\text{ATP} + \text{Oxalacetate} \rightleftharpoons \text{Phosphoenolpyruvate} + \text{ADP} + \text{CO}_2 \qquad 6.$$

may explain the phenomenon observed by Mudge *et al.* α-Ketoglutarate can give rise both to oxalacetate and to ATP (by transphosphorylation of ADP with GTP) even in presence of high levels of DNP. The occurrence of intra-mitochondrial GTP and ATP is indicated by recent work (136, 169). Bartley (170) has also observed the formation of phosphoenolpyruvate with particles of sheep kidney.

Lindberg & Ernster (171) have discovered an interesting antagonism

[14] Similar systems have been found in plants by Bandurski & Greiner (167) and by Vennesland *et al.* (168).

between calcium and manganese ions. Rat liver mitochondria lose the capacity to carry out oxidations of the citric acid cycle in presence of calcium ions particularly when the level of ATP is kept at a minimum (this obtains in presence of the glucose-hexokinase system). DPN can restore the capacity for oxidation but not for oxidative phosphorylation. However, DPN supplemented with ATP and manganous ions restores both oxidation and phosphorylation to the level obtaining in absence of added calcium. Lindberg & Ernster suggest that the oxidation-reduction catalysts of the electron transport system which are involved in oxidative phosphorylation can exist in two forms, only one of which can participate in phosphorylation but either of which is active in electron transport.

Fatty Acid Oxidation and Synthesis

Several recent reviews dealing with the complete reconstruction of the fatty acid oxidation cycle with combinations of individual, separated enzymes have appeared during 1953 to 1954 (172 to 175). In addition the essential facts have been reported in last year's review (79, 176) and F. Lynen will be covering the subject in the chapter on Lipide Metabolism in the present volume. It will be, therefore, superfluous to repeat those details which have been presented elsewhere. Some consideration will, however, be given here to some aspects of fatty acid oxidation and synthesis which are still unresolved.

Two flavoproteins have been isolated in homogeneous state both of which catalyze the oxidation of fatty acyl CoAs to their corresponding unsaturated derivatives although there is considerable difference in specificity with respect to the chain-length of the fatty acid. The green dehydrogenase (130) works on acyl CoAs from C_4 to C_8 while the yellow dehydrogenase is active on all acyl CoAs from C_4 to C_{16} (131, 172). The affinity of the former increases as the chain length decreases while that of the latter decreases as the chain length decreases. Clearly these properties suggest an equitable distribution of labor, the yellow dehydrogenase degrading the fatty acyl CoAs down to about C_8 and the green enzyme taking over from there on in. In this way an orderly process of degradation of the fatty acid becomes mandatory.

The green and yellow dehydrogenases occur in tissues in close association and are only separable into the individual component parts by rather drastic treatment. This association and combination of enzymes which attack respectively the long and short chain fatty acids and their derivatives may be quite general, and the same logic would apply to such an arrangement for the other enzymes of the fatty acid oxidation cycle as discussed above for the acyl CoA dehydrogenases. Two enzymes have been previously demonstrated to collaborate in catalyzing the acylation of CoA, one specific for short chain fatty acids and derivatives (C_4 to C_{12}) (177) and a second one apparently specific for the long chain derivatives (C_8 to C_{18}) (178).

The question whether synthesis of long chain fatty acids proceeds by the reversal of the oxidative pathway has now become the focus of much atten-

tion. The paradox is that each step of the fatty acid oxidation cycle has indeed been shown to be reversible. Yet Stansly & Beinert (179) found that in a system containing all the so far recognized individual enzymes they could convert acetyl CoA to butyryl CoA but not to any higher derivative. The difficulty seems to resolve itself into the following terms. The particular β-ketoacyl CoA cleavage enzyme used by the Wisconsin group which catalyzes the general reaction

$$\beta\text{-Ketoacyl CoA} + \text{CoA} \rightleftarrows \text{Acetyl CoA} + \text{Acyl CoA} \qquad 7.$$

has the property that when presented with a mixture of acetyl CoA and butyryl CoA will catalyze exclusively reaction 8 and not 9:

$$\text{Acetyl CoA} + \text{Acetyl CoA} \rightleftarrows \text{Acetoacetyl CoA} + \text{CoA} \qquad 8.$$

$$\text{Acetyl CoA} + \text{Butyryl CoA} \rightleftarrows \beta\text{-Ketocaproyl CoA} + \text{CoA}. \qquad 9.$$

Clearly a different cleavage enzyme must operate in synthesis with the reverse specificity, i.e., higher affinity for the long chain fatty acyl CoAs than for acetyl CoA.

A second problem posed by efforts to reconstruct a system of soluble enzymes for synthesis of long chain fatty acids concerns the reversal of the first oxidative step. Although a specific enzymatic link of the fatty acyl CoA dehydrogenases to cytochrome-*c* has been found (129), as discussed in a previous section, it is not known whether cytochrome-*c* is the physiological oxidant for the oxidation of fatty acyl CoAs; most likely the oxidant is one of the hemoproteins of the terminal electron transport system. This gap in our knowledge complicates the task of arranging for the reversal of the first oxidative step, a reversal which is necessary in synthesis. From what is known about fatty acid synthesis in extracts of *Clostridium kluyveri* (180, 181) it seems quite certain that DPNH is the ultimate electron donor. Since DPN is not an electron acceptor for the dehydrogenases, DPNH cannot act directly as the electron donor in the reverse direction. There is thus clear indication that either some carrier shuttles between DPNH and the reduced acyl CoA dehydrogenases or some device, perhaps a metal, is available for linking DPNH dehydrogenase to the fatty acyl CoA dehydrogenases.

The laboratories of Gurin (182) in this country and of Popják (183) in England have succeeded in effecting fatty acid synthesis in soluble systems from liver and mammary tissue respectively. Acetate is incorporated into fatty acids. DPN, ATP, and CoA requirements are demonstrable in both these systems.

In higher plants the mechanisms for fatty acid oxidation seem to be different from those in animal tissues. Stumpf and his collaborators (20) have recognized two different enzyme systems that participate in the oxidative breakdown of palmitate in germinating peanut cotyledons. The possibility that these two systems act in a complementary fashion cannot as yet be excluded. One system which is associated with microsomal particles catalyzes the oxidation of palmitate by DPN with liberation of CO_2. This appears to be a stepwise, serial oxidation down the carbon chain since labeled CO_2 is

formed from palmitate labeled in carbon atoms 2, 3, and 11. However, this oxidative breakdown is not typical for β-oxidation since neither CoA nor the citric acid cycle is involved. The second system (184) which has been obtained in soluble form (and which may be identical with a corresponding enzyme system also found in microsomes) catalyzes the oxidative decarboxylation of fatty acids from C_{14} to C_{18} with the α-keto fatty acid as the probable intermediate. Only the carboxyl labeled fatty acids yield labeled CO_2 in this process, a fact which suggests that the substrate of the enzyme is not oxidized successively. Glycolic acid and to a lesser extent L-lactic acid stimulate considerably the CO_2 production by the soluble fatty acid oxidizing system. Franke & Frehse (185) have studied the over-all pattern of fatty acid oxidation in a variety of seeds and plants.

Pyridinoproteins and Pyridine Nucleotides[15]

The year's crop of new pyridinoprotein enzymes or of new variants of classical types has been a very abundant one. Table I contains a summary of the salient features of these recent additions to the category of pyridinoprotein enzymes. Many pyridinoprotein enzymes turn up in two forms, one specific for DPN and the other for TPN. Plaut & Sung (187) have described a DPN specific isocitric dehydrogenase in heart muscle which unlike the yeast counterpart does not require the presence of adenylic acid. Arnon *et al.* (188) find not only that there is a TPN specific glyceraldehyde phosphate dehydrogenase in photosynthetic tissues but also that there are two varieties of such, one of which requires inorganic phosphate while the other does not.

Sufficient information is now at hand about the prosthetic groups of enzymes to warrant the conclusion that the pyridine nucleotide coenzymes are indeed the work-horses of biological oxidations. Four of the five oxidative steps in the citric acid cycle, the two oxidative steps in glycolysis or fermentation, one of the two oxidative steps in fatty acid oxidation, a key oxidative reaction in pyrimidine synthesis (189), several oxidative steps in steroid transformations (190 to 193), all the oxidative steps in the glucose to tetrose sequence of reactions (194, 195), the oxidation of dithiolipoic to the closed ring form (196), and the transformation of dihydrofolic to tetrahydrofolic acid (197, 198) all require the presence of one or the other pyridine nucleotide.

Harting & Velick (224) have made additional contributions to their systematic studies of glyceraldehyde-3-phosphate dehydrogenase particularly with reference to the transfer of the acetyl group of acetyl phosphate to various acceptors. This transfer requires the presence of bound DPN, but the DPN does not function in this process as a hydrogen donor or acceptor. Hayes & Velick (225) have considered the number and strength of coenzyme (DPN and DPNH) binding sites in yeast alcohol dehydrogenase.

Several new enzymatic mechanisms for oxidation of DPNH have been uncovered particularly in plant tissues. Peas (226), cucumber (227), and

[15] Cf. Singer & Kearney (1,186) for an excellent review of the present knowledge in this field.

yeast (228) have been shown to contain a DPNH dehydrogenase which catalyzes the oxidation of DPNH by some oxidation product of ascorbic acid which is probably at an oxidation level between that of ascorbic and dehydroascorbic acid. This product can be formed from ascorbic acid either enzymatically with the aid of ascorbic oxidase or nonenzymatically with cupric or ferric salts as catalysts. A DPNH specific cysteine reductase has been demonstrated in pea seeds and yeast by Romano & Nickerson (229). Furthermore higher plants contain an enzyme system which catalyzes the oxidation of DPNH by diketosuccinate with α-keto-β-hydroxysuccinate as the probable reduction product (230). Extracts of *Streptococcus faecalis* contain a cyanide-insensitive peroxidase (231) which catalyzes the reaction:

$$H_2O_2 + DPNH + H^+ \rightarrow 2\ H_2O + DPN^+. \qquad 10.$$

An enzyme from *A. aerogenes* or *S. aureus* reduces diacetyl to acetoin by DPNH (209).

Huennekens & Gabrio (232) have isolated from hemolysates an enzyme which catalyses the oxidation of TPNH by methemoglobin or oxygen when supplemented with methylene blue or a naturally occurring unidentified cofactor. An enzyme of similar properties, which acts as DPNH oxidase or diaphorase, has been isolated from pig heart by Huennekens *et al.* (233). The remarkable feature in the mechanism of catalysis by these enzymes seems to be that flavins evidently are not involved. Williams (234) has confirmed the finding of Reif & Potter (235) of an antimycin insensitive pathway for DPNH oxidation in animal tissues. An interesting new development is the report of the requirement of both DPNH (236, 237, 238) and FMN[3] (238) for the light emitting oxidation of bacterial luciferin.

The stereospecific reduction of DPN in pyridinoprotein enzyme systems has been extensively studied by Vennesland & Westheimer (239). Yeast alcohol dehydrogenase, muscle lactic dehydrogenase, and wheat germ malic dehydrogenase transfer hydrogen to the paracarbon atom of the pyridine ring by attack from one and the same side of the plane of the ring. However, in the bacterial transhydrogenase reaction (240) and in the transfer of hydrogen by β-hydroxysteroid dehydrogenase (241) the transfer takes place from the opposite side.

During the last few years the group at the McCollum Pratt Institute has conducted a wide survey of the chemistry and the enzymatic transformations of the pyridine nucleotides. This survey has included a study of (*a*) transhydrogenations between pyridine nucleotides (240, 242, 243, 244), (*b*) the position of the carbon in the pyridine ring to which hydrogen is added during reduction (245, 246), (*c*) the nonenzymatic formation of addition compounds between DPN and cyanide (247), dihydroxyacetone (248) and hydroxylamine (249), (*d*) the nature of the yellow intermediate formed during the reduction of DPN by dithionite (250), (*e*) the inhibition of liver and yeast alcohol dehydrogenases by hydroxylamine and cyanide (251, 252, 253), (*f*) the availability of enzyme bound DPN for other enzymatic reactions (254), (g) the enzymatic synthesis of TPN from DPN (260, 261), (*h*) the degrada-

TABLE I

PYRIDINE NUCLEOTIDE LINKED DEHYDROGENASES RECENTLY DESCRIBED*

Name	Reaction Catalyzed	Nucleotide Specificity	Source	Remarks	Ref
Glycerol dehydrogenase	Glycerol $\overset{DPN}{\rightleftharpoons}$ dihydroxy acetone	not reported	*Bacillus subtilis*		199
		DPN	*Aerobacter aerogenes*		200
	Glycerol $\overset{DPN}{\rightleftharpoons}$ D,L-glyceraldehyde	DPN	*Escherichia coli*	15- to 20-fold purification	201
		DPN	rat liver		202
Glyceraldehyde phosphate dehydrogenase	Glyceraldehyde-3-phosphate $\xrightarrow{TPN}$ glyceric acid-3-phosphate	TPN	sugar beet leaves	90- fold purification; no phosphate requirement	188
D-glyceric acid dehydrogenase	D-glyceric acid $\overset{DPN}{\rightleftharpoons}$ hydroxypyruvic acid	DPN	parsley leaves		203
Polyol dehydrogenase (ketose reductase)	Acyclic polyol $\overset{DPN}{\rightleftharpoons}$ ketose (L-iditol, L-arabitol)	DPN	rat liver	70-fold purification; enzymes with different specificity are present in microorganisms	204 205 (cf. 206)
Inositol dehydrogenase	*Myo*-inositol $\overset{DPN}{\rightleftharpoons}$ 2-keto-*myo*-inositol	DPN	*A. aerogenes*		207
Alcohol dehydrogenase	Alcohol $\overset{TPN}{\rightleftharpoons}$ aldehyde	TPN	*Leuconostoc mesenteroides*	8-fold purification	208
Butylene glycol dehydrogenase	Butylene glycol $\overset{DPN}{\rightleftharpoons}$ acetoin	DPN	*A. aerogenes* *Staphylococcus aureus*	80-fold purification	209 (cf. 210)

TABLE I—(*continued*)

Name	Reaction Catalyzed	Nucleotide Specificity	Source	Remarks	Ref
Histidinol dehydrogenase	Histidinol $\xrightarrow{2\ DPN}$ histidine	DPN	Soil bacterium, yeast, *E. coli*	8- to 14-fold purification; probably two step process	211
Formaldehyde dehydrogenase	Formaldehyde $\xrightarrow{DPN}$ formic acid	DPN	beef liver	160-fold purification; activated by glutathione	212
Betaine aldehyde dehydrogenase	Betaine aldehyde $\xrightarrow{DPN}$ betaine	DPN	rat liver	5-fold purification	213
Benzaldehyde dehydrogenase	Benzaldehyde $\overset{DPN}{\rightleftharpoons}$ benzoic acid	DPN	*Pseudomonas fluorescens*	2 separate enzymes; acetaldehyde, glyceraldehyde and methylglyoxal not oxidized	214
	Benzaldehyde $\overset{TPN}{\rightleftharpoons}$ benzoic acid	TPN	*Pseudomonas fluorescens*		
Isocitric dehydrogenase	D-Isocitric acid $\xrightarrow{DPN}$ α-ketoglutaric acid + CO_2	DPN	beef heart	40- to 50-fold purification	187
Dihydroorotic dehydrogenase	Dihydroorotic acid $\overset{DPN}{\rightleftharpoons}$ orotic acid	DPN	Soil bacterium	7-fold purification	189
Quinic dehydrogenase	Quinic acid $\overset{DPN}{\rightleftharpoons}$ 5-dehydroquinic acid	DPN	*A. aerogenes*	9-fold purification	215
Shikimic dehydrogenase	Shikimic acid $\overset{TPN}{\rightleftharpoons}$ 5-dehydroshikimic acid	TPN	*A. aerogenes*		215

TABLE I—(*continued*)

Name	Reaction Catalyzed	Nucleotide Specificity	Source	Remarks	Ref
Lipoic dehydrogenase	Lipoic $(SH)_2 \overset{DPN}{\rightleftharpoons}$ Lipoic S_2	not reported	*E. coli*	200-fold purification	196
Ammonium dehydrogenase	$NH_4OH \overset{DPN}{\rightleftharpoons} NH_2OH$	DPN TPN, DPN	*B. subtilis* *Neurospora crassa*	Slightly active with TPN 10 fold purification	216 217
β-Hydroxysteroid dehydrogenase	3β(17β)-Hydroxysteroid $\rightleftharpoons$ 3(17)-ketosteroid	DPN	*Pseudomonas*	25- to 50-fold purification	190
3α-Hydroxysteroid dehydrogenase	3α-Hydroxysteroid $\overset{DPN}{\rightleftharpoons}$ 3-ketosteroid	not reported	*Pseudomonas*	30- to 50-fold purification	191
The implication of pyridine nucleotides in the following reactions has been shown					
	7,8-Dihydrofolic acid $\xrightarrow{DPNH}$ 5,6,7,8-tetrahydrofolic acid	not reported	pigeon liver		197, 198 (cf. 218, 219)
	N-5-formyl-5,6,7,8-tetrahydrofolic acid $\xrightarrow{DPNH}$ N-5-hydroxymethyl-5,6,7,8-tetrahydrofolic acid	not reported	pigeon liver	suggested	198

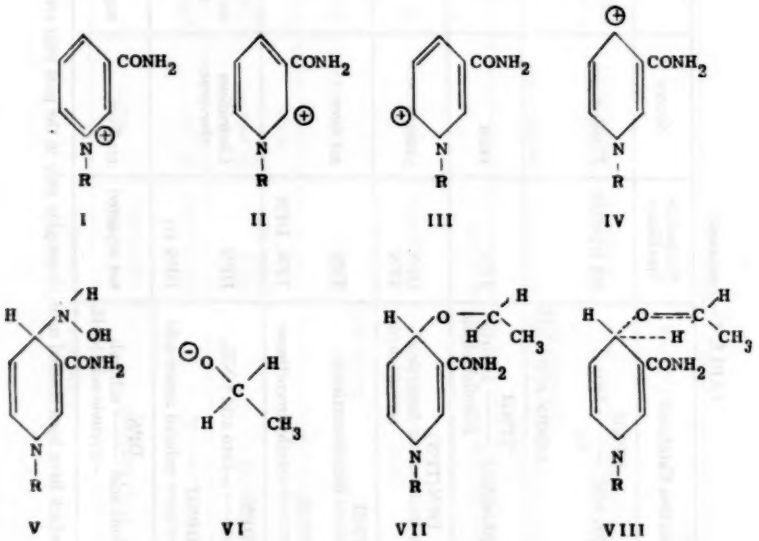
TABLE I—(*continued*)

Name	Reaction Catalyzed	Nucleotide Specificity	Source	Remarks	Ref
	Imidazole acetic acid $\xrightarrow[O_2]{DPNH}$ formyl-aspartic acid+NH_3	not reported	*Pseudomonas*		220
	β-L-Aspartyl phosphate $\xrightarrow{TPNH}$ aspartic-β-semialdehyde	TPN	yeast		221
	L-Homoserine $\overset{DPN(TPN)}{\rightleftharpoons}$ Aspartic-β-semialdehyde	DPN TPN	yeast		221
	Cortisone $\xrightarrow{TPNH}$ dihydrocortisone	TPN	rat liver		192
	$\overset{TPNH(DPNH)}{\rightleftharpoons}$ tetrahydrocortisone	TPN, DPN			
	Amino acid $\xrightarrow{DPN}$ α-keto acid+NH_3	DPN	*Clostridium sporogenes*	see text (Strickland reaction)	222, 223
	Amino acid $\xrightarrow{DPNH}$ reduced amino acid	DPN (?)			
	L-cysteinesulfinic acid $\xrightarrow{DPN}$ sulfinyl-pyruvic acid+NH_3	not reported	rat liver	suggested	303

* Pyridinoproteins which are known but which have been studied more thoroughly only in the past year are included in the table.

tion of pyridine nucleotides by alkali (255), diphosphopyridine nucleotidase (256), and pyrophosphatase (253), and (*i*) the synthesis of structural analogues of DPN (257, 258, 259). On the basis of evidence drawn from these many different lines of investigation, Kaplan and his group have now constructed a composite picture of the molecular mechanism by which the reducing substrate is oxidized by DPN in pyridinoprotein enzyme systems.

An essential piece of information about pyridine nucleotides that has been missing until very recently is the knowledge of the carbon atom in the pyridine ring which is hydrogenated during enzymatic reduction. The answer has now been supplied by studies of Colowick's group (245, 246). DPN



was reduced by dideuteroethanol in presence of yeast alcohol dehydrogenase. The labeled, reduced DPN was oxidized back to DPN with ferricyanide and then was degraded to nicotinamide by means of DPN nucleotidase. The methylated derivative of nicotinamide was formed and oxidized to a mixture of the 2- and 6-pyridones which were then separated from one another. If the atoms of deuterium were located in the para position both pyridones would contain equal amounts of label. Since labeling in the two pyridones was found to be identical, Colowick *et al.* have concluded that DPN is reduced in the para position exclusively during enzymatic reduction.

DPN forms addition compounds with cyanide, bisulfite, dihydroxyacetone (and other ketones and aldehydes), and hydroxylamine (247, 248, 249).

In all cases the addition compounds resemble DPNH both in spectroscopic and chemical properties. The addition reaction leads to the same change in ring structure as reduction, viz., the change to a para-dihydropyridine structure (cf. structure V). The resonance form of DPN which plays a dominant role in these reactions is considered to be the carbonium structure IV. For practical purposes the para carbon atom of the ring may be said to behave like a carbonyl carbon.

When DPN is reduced by dithionite in bicarbonate solution a yellow intermediate appears. It has long been assumed that this compound is a semiquinone which yields DPNH by dismutation. The experiments of Yarmolinsky & Colowick (250) favor the conclusion that the first step in the reduction of DPN by dithionite is again an addition reaction. Evidence is presented that the intermediate is the para sulfoxylate of DPN, i.e., DPN-OSONa.

The next important development came during a study of the inhibition of horse liver alcohol dehydrogenase (ADH) by hydroxylamine (251, 252, 253). When DPN and hydroxylamine are incubated together in presence of ADH the rate of oxidation of alcohol by DPN is inhibited almost completely. In absence of the enzyme or with DPNH instead of DPN this effect is not observed. High levels of alcohol reverse the inhibition competitively. Analysis of the inhibition has led Kaplan & Ciotti (251) to the conclusion that enzyme bound DPN can react readily with hydroxylamine and that hydroxylamine is probably occupying the same site on the enzyme as the substrate of horse liver ADH. There are two intriguing aspects of the inhibition by hydroxylamine. First hydroxylamine is substituting for ethanol, the reducing substrate of the enzyme. Hence a knowledge of how hydroxylamine combines with enzyme-bound DPN might also be applicable to the mode of combination of the reducing substrate with the enzyme. Secondly the nonenzymatic interaction of hydroxylamine with DPN takes place only at alkaline pH, whereas the combination with enzyme bound DPN can take place at neutral pH. It seems therefore that the structure of DPN has become modified by virtue of the combination with enzyme in favor of IV. Similarly binding of hydroxylamine to the enzyme may stabilize the anionic form. The net effect is that a rapid reaction between DPN and hydroxylamine can go on at neutral pH.

On the basis of analogy drawn from the chemical literature dealing with isoquinoline derivatives Burton & Kaplan (249) have proposed the following mechanism for the interaction of enzyme-bound DPN with hydroxylamine and ethanol: DPN is assumed to exist in four resonating forms (Formulae I to IV) of which structure IV, which appears to carry the greatest positive charge, is most likely to combine with the anionic form of hydroxylamine ($^-$NHOH) to give the product represented by Formula V. In an analogous fashion DPN in the form of structure IV is thought to combine with the anionic form of ethanol (Formula VI) to give the product represented by VII. Obviously the ionization of ethanol proceeds only in combination with the enzyme. The DPN-ethanol addition compound then undergoes trans-

formation to the transition compound VIII in which there is, in effect, a shift of the hydrogen atom (to be transferred to the ring) towards the ring and a corresponding shift of the ethanolic oxygen away from the ring. Finally VIII breaks up into DPNH and acetaldehyde. In the enzymatic interaction of DPN with reducing substrate the first step is thus visualized as an addition reaction. The demonstration by Westheimer, Vennesland and their group of a direct transfer of a hydrogen atom from reducing substrate to DPN is in line with this hypothesis.

Rafter *et al.* (262) have described the enzymatic conversion of DPNH to an enzymatically inactive compound which no longer shows the characteristic band at 340 mμ. This conversion is catalyzed by the triosephosphate dehydrogenase.

Leach *et al.* (263, 264, 265) have constructed model dehydrogenase systems for the study of hydrogen transfer. These authors develop the theme that the meaning of the precise order in which the various catalysts intervene in the electron transport chain is not only to be found in terms of oxidation-reduction potential but also in terms of mechanism. The sequence of steps would then make possible a merger between an exclusive hydrogen transfer mechanism at the substrate end and an exclusive electron transfer mechanism at the oxygen end.

Mahler & Elowe (266) have studied the oxidation of bound DPNH (bound to the triosephosphate dehydrogenase) by DPNH cytochrome-*c* reductase with cytochrome-*c* or indophenol as electron acceptor. The reduction rates are of an order of magnitude comparable to that with free DPNH under similar experimental conditions and thus the possibility of the dissociation of the DPNH-enzyme complex is excluded. This phenomenon may have important implications for interactions between the DPNH forming systems and the DPNH oxidizing system of mitochondria. Differences in the availability to other enzymes of oxidized or reduced DPN bound to active (SH) or inactive (S-S) triosephosphate dehydrogenase have been reported by Astrachan (254). Rodkey (267) has measured the E_0' of the system DPNH−DPN at pH 7.0 and 30°C. and reports a value of -0.318 ± 0.003 volts, in essential agreement with the value recently reported by Burton & Wilson (268).

Stickland in 1934 (269) and later Woods (270) in 1936 showed that *C. sporogenes* was able to grow by utilization of reactions in which certain amino acids were oxidized by reduction of other amino acids. The amino acids which served as electron donors were alanine, valine, and leucine while the amino acids which served as electron acceptors were glycine, proline, and hydroxyproline. These interactions have been more thoroughly analyzed by Mamelak & Quastel (222) and by Nisman and his collaborators (271) [cf. also (223)]. They can be readily conceived of as DPN mediated reactions between two pyridino-protein enzyme systems. For example the reaction between alanine and proline can be represented as follows:

$$\text{alanine} + \text{DPN}^+ + \text{H}_2\text{O} \rightarrow \text{pyruvic acid} + \text{NH}_3 + \text{DPNH} + \text{H}^+ \quad 11.$$
$$\text{DPNH} + \text{proline} + \text{H}^+ \rightarrow \delta\text{-amino valeric acid} + \text{DPN}^+ \quad 12.$$

$$\text{alanine} + \text{proline} + \text{H}_2\text{O} \rightarrow \text{pyruvic acid} + \text{NH}_3 + \delta\text{-amino valeric acid} \quad 13.$$

In crude extracts pyruvate does not accumulate since it is degraded by an oxidase system in conjunction with transacetylase according to the equation:

$$\text{pyruvate} + \text{DPN}^+ + \text{CoA} \rightarrow \text{acetyl CoA} + \text{DPNH} + \text{H}^+ + \text{CO}_2 \quad 14.$$
$$\text{acetyl CoA} + \text{P}_i \rightarrow \text{acetyl phosphate} + \text{CoA} \quad 15.$$

It remains to be determined how many specific pyridinoprotein enzymes carry out reactions analogous to those of equations 11 and 12.

Oxidation of Pyruvate

In bacteria pyruvic acid is oxidized in at least four different ways as shown below, the first line in each scheme indicating the path of electrons and the subsequent lines showing the reaction sequence:

(A) $\text{Pyruvate} \rightarrow \text{Lipoic} \rightarrow \text{DPN}$

$$\text{Pyruvate} + \text{DPN} + \text{CoA} \rightarrow \text{Acetyl CoA} + \text{DPNH} + \text{CO}_2$$
$$\text{Pyruvate} + \text{DPN} + \text{P}_i \xrightarrow{\text{CoA}} \text{Acetyl phosphate} + \text{DPNH} + \text{CO}_2$$

(B) $\text{Pyruvate} \rightarrow \text{Flavin} \rightarrow \text{O}_2$

$$\text{Pyruvate} + \text{O}_2 + \text{P}_i \rightarrow \text{Acetyl phosphate} + \text{CO}_2 + \text{H}_2\text{O}_2$$
$$\text{Pyruvate} + \text{dye} + \text{P}_i \rightarrow \text{Acetyl phosphate} + \text{CO}_2 + \text{reduced dye}$$

(C) $\text{Pyruvate} \rightarrow [\text{diphosphothiamine}]? \rightarrow [\text{cytochromes}] \rightarrow \text{CO}_2$

$$\text{Pyruvate} + \tfrac{1}{2}\text{O}_2 \rightarrow \text{Acetate} + \text{CO}_2$$

(D) $\text{Acetaldehyde} \rightarrow [?] \rightarrow \text{O}_2$

$$\text{Pyruvate} \rightarrow \text{Acetaldehyde} + \text{CO}_2$$
$$\text{Acetaldehyde} + [?] \rightarrow \text{Acetate} + \text{reduced } [?]$$

Diphosphothiamine is an essential coenzyme in all four mechanisms. Mechanism A is the classical mechanism established in animal tissues and some species of bacteria by Reed (272), Gunsalus (273, 274), Schweet & Cheslock (275), and Ochoa, Korkes and their collaborators (276). This mechanism has been reviewed *in extenso* in earlier volumes, (65, 79) and in three symposia (272, 274, 277, 278). Strecker & Ochoa (279) have recently established that the pyruvic oxidase system of *S. aureus* and *A. aerogenes* conforms in all respects to the prototype of mechanism A as found in *E. coli*. That is to say the system contains two enzymes, one which catalyzes the oxidation of pyruvate to acetyl CoA while the other catalyzes the oxidation of reduced lipoic acid by DPN. Nisman & Wiesendanger (280, 281) also have established the A mechanism as applicable to the pyruvic oxidase systems of *C. sporogenes* and *C. saccharobutyricum*. A curious ATP requirement was found in the latter case. Hager *et al.* (282) have presented new evidence that mechanism B applies to the pyruvic oxidase system of *Lactobacillus delbrueckii*. Flavin takes the place of lipoic acid as primary electron acceptor and because of its autoxidizability can transfer electrons to molecular oxygen. However, the nature of the primary acyl acceptor in this system, whether

diphosphothiamine or flavin, is still unresolved. On the basis of analogy with other systems one would assume that the formation of acetylphosphate involves the transfer of an acetyl group from the pyruvic oxidase to inorganic phosphate by a reaction in which CoA and transacetylase participate. As long as the complete absence of enzyme bound CoA is not demonstrated this possibility does not seem to be excluded. O'Kane (283) has studied a pyruvic oxidase system in *C. butyricum* which can best be described in terms of mechanism B although the author does not implicate flavin in the catalysis. CoA and ferrous ions are required in this system.

It is postulated by O'Kane (283) that the oxidation of pyruvate to acetate in extracts of *Proteus vulgaris* proceeds according to mechanism C. The role of diphosphothiamine as electron acceptor would be indeed an innovation for which more compelling evidence would be required than has been presented.

The presence of a carboxylase in *Acetobacter suboxydans* by King & Cheldelin (284) provides the basis for mechanism D. In this mechanism the decarboxylation and the subsequent oxidation of acetaldehyde are separable processes catalyzed by different enzymes.

Other Pathways to Active Acetate

The ATP-catalyzed conversion of acetate to an active form is also a process which has at least two variants. The classical reaction elucidated by Jones *et al.* (285), Lynen (173), Beinert *et al.* (286), Von Korff (287), Hele (288), and Millerd & Bonner (289) involves the reaction:

$$\text{ATP} + \text{acetate} + \text{CoA} \rightleftharpoons \text{Acetyl CoA} + \text{AMP} + \text{P-P}_i. \qquad 16.$$

Rose *et al.* (290) have discovered an acetokinase system from *S. hemolyticus* and *E. coli* which catalyzes the reaction:

$$\text{ATP} + \text{acetate} \rightleftharpoons \text{ADP} + \text{acetyl phosphate}. \qquad 17.$$

This is in effect a reversible transfer of a phosphate group from ATP to acetate. CoA appears to play no role in this bacterial acetate activation enzyme system. Seaman & Naschke (291) claim that in *Tetrahymena* a CoA and ATP dependent direct pathway exists from succinate to acetyl CoA in which neither acetate nor acetyl phosphate appears to be an intermediate and the participation of the Krebs cycle seems to be excluded.

Citric Acid Cycle

Three new ways of splitting citric or isocitric acid have been discovered, one in pigeon liver by Srere & Lipmann (292), the other two in bacterial extracts independently by several investigators. The liver system catalyzes the reversible reaction:

$$\text{ATP} + \text{citrate} + \text{CoA} \rightleftharpoons \text{Acetyl CoA} + \text{oxalacetate} + \text{ADP} + \text{P}_i. \qquad 18.$$

This can be looked upon as a variation of the Ochoa-Stern (293) condensation reaction. In addition to the condensation of acetyl CoA and oxalacetate to form citrate there is concomitant synthesis of ATP from ADP and P_i.

The thioester linkage of acetyl CoA is eventually replaced by the P–O–P linkage of ATP presumably by a series of replacement reactions.

A widely distributed system in bacteria called isocitritase (294) or isocitric lyase (295, 296, cf. 297) catalyzes the cleavage of D-isocitrate according to the equation:

$$\text{D-isocitrate} \rightarrow \text{glyoxylate} + \text{succinate.} \qquad 19.$$

CoA plays no role in this process. However, the presence of magnesium ions is essential. In other bacteria such as *S. faecalis* and *E. coli*, Gunsalus and his collaborators (298, 299) have found evidence of an enzyme (citritase) which catalyzes the reaction:

$$\text{Citrate} \rightarrow \text{Oxalacetate} + \text{acetate.} \qquad 20.$$

Again coenzyme A is not required for the cleavage reaction.

Shemin & Russell (300) have proposed a new cycle geared to the citric acid cycle which in effect brings about the oxidation of glycine to carbon dioxide, water, and NH_3. This cycle may be represented as below in Figure 1.

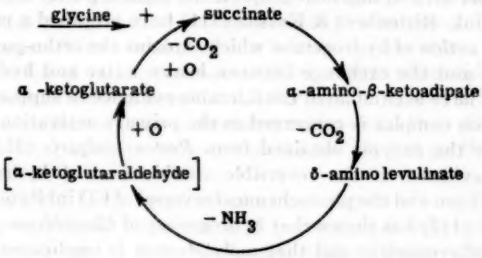


FIG. 1. The proposed Shemin cycle.

The complete oxidation of glycine is perhaps only a coincidence. The main function of the cycle appears to be that of generating key intermediates for the synthesis of both porphyrins and purines, δ-amino levulinate for synthesis of porphyrins (300 to 302) and the still hypothetical α-ketoglutaraldehyde for synthesis of purines. As yet this Shemin cycle has not been established at the enzyme level, representing at present merely an interpretation of isotopic studies. That succinate condenses with glycine is quite certain but whether in the form of succinyl CoA or any other active form of succinate is still unknown.

OXIDATION OF CYSTEINESULFINATE AND SULFITE

The enzymatic processes by which L-cysteinesulfinate is converted to pyruvate and sulfate have become clarified by the recent studies of Singer & Kearney (303, 304) and Fromageot and collaborators (305). In various animal tissues and in *Proteus vulgaris* the oxidation of L-cysteinesulfinate ($COOH \cdot CHNH_2 \cdot CH_2 \cdot SOO^-$) proceeds by way of transamination with either α-ketoglutarate or oxalacetate. β-Sulfinylpyruvate, the primary prod-

uct of transamination, does not accumulate but undergoes cleavage to pyruvate and sulfite. Finally an enzyme, which was also studied in Handler's laboratory (306) and appears to be a flavoprotein (307), catalyzes the oxidation of sulfite to sulfate. A minor pathway present in rat liver involves the DPN-dependent oxidation of L-cysteinesulfinate to β-sulfinylpyruvate which then further decomposes. Chapeville & Fromageot (308) could show incorporation of labeled SO_2 into cysteinesulfinate in presence of pyruvate and glutamate with a preparation from rabbit kidney acetone powder and were thus evidently able to reverse the cleavage of β-sulfinyl pyruvate into pyruvate and SO_2. This would be analogous to fixation of CO_2 into pyruvate.

Hydrogenase[16]

Much progress has been made in the study of the bacterial enzyme which catalyzes the oxidation of hydrogen by various electron acceptors. Sadana & Jagannathan (310) have solubilized the enzyme of *Desulphovibrio desulphuricans* and brought it to a state of very high purity ($Q_{H_2}^{\text{methylene blue}}$ of 2.5×10^6 μl per hr. and mg. nitrogen). At the highest purity level the enzyme solution is pink. Rittenberg & Krasna (311) have proposed a mechanism for the mode of action of hydrogenase which explains the ortho-para conversion of hydrogen and the exchange between heavy water and hydrogen. These authors also have accumulated considerable evidence in support of the view that some iron complex is concerned in the primary activation of molecular hydrogen by the enzyme obtained from *Proteus vulgaris* (312). The most compelling evidence is the reversible combination of hydrogenase with molecular oxygen and the photochemical reversal of CO inhibition. The study of Shug *et al.* (117) has shown that hydrogenase of *Clostridium pasteurianum* is a molybdoflavoprotein and that molybdenum is implicated in the interaction of the reduced flavoprotein enzyme with one-electron acceptors such as cytochrome-*c* and ferricyanide, and with special acceptors like nitrate. If it is permissible to combine the results of the studies on hydrogenase obtained from three different sources, hydrogenase would appear to be a flavoprotein chelated with both iron and molybdenum, the former metal being involved in the primary activation process and the latter in electron transfer to special acceptors.

Oxidizing Enzymes

Consideration of oxidizing enzymes strictly according to a systematic or logical classification is a counsel of perfection for a review which has to fit into a strait jacket of a fixed number of pages. The present section is a catchall for some newly described oxidizing enzymes which can not be fitted into the major categories discussed above.

Sutton (313) has purified the lactic oxidizing enzyme of *Mycobacterium phlei* previously discovered by Edson (314, 315) and studied by Yamamura *et al.* (316) which catalyzes the oxidative decarboxylation of lactate to ace-

[16] Cf. Gest (309) for a review on hydrogenase.

tate and CO_2. The two activities, i.e., the oxidation and the decarboxylation, both appear to be associated with the same functional unit. Pyruvate is not the intermediate in the oxidative conversion of lactate to acetate. This lactic oxidase is reminiscent of the malic enzyme of Ochoa *et al.* (317) which catalyzes the oxidative decarboxylation of malate to pyruvate and CO_2 without intermediary formation of oxalacetate. No knowledge of the prosthetic groups is yet available other than that flavin is absent and cyanide is not an inhibitor of the enzyme.

The discovery of a glycolic oxidase in plants (318, 319, 319a) has now been followed by the isolation of a corresponding flavoprotein enzyme from rat liver by Kun *et al.* (320). The oxidase catalyzes the oxidation of glycolic acid to glyoxylic acid by molecular oxygen with formation of H_2O_2. The flavoprotein can be converted into an apoenzyme by precipitation with acid ammonium sulfate. Activity can then be restored by addition of either FMN or FAD[2].

Much effort has been directed in the past to the study of amine oxidase in animal tissues. The particulate nature of the oxidase in liver, kidney, and other tissues has perforce complicated the task of purification. Tabor *et al.* (321) have prepared from beef plasma a soluble amine oxidase which catalyzes the oxidation of various amines to their corresponding aldehydes with concomitant formation of ammonia and H_2O_2. The substrate specificity is not identical with that of liver monoamine oxidase, and there are other lines of evidence in favor of the nonidentity of the soluble and particulate amine oxidases [cf. Werle & Roewer (322)].

Satake & Fujita (323) obtained cell-free extracts of an *Achromobacter* species which contained histamine and putrescine dehydrogenases. Both these enzymes are adaptive. By prolonged dialysis these enzymes can be partly inactivated and activity can be restored by addition of FAD.

N-methylamino acids are oxidized by an enzyme prepared from rabbit kidney (324) which catalyzes the reaction:

$$\text{N-Methylamino acid} + O_2 + H_2O \rightarrow \text{Amino acid} + \text{Formaldehyde} + H_2O_2. \quad 21.$$

Moritani *et al.* find that only N-mono-methyl amino acids of the L-series are attacked by the enzyme. The presence of an aromatic or heterocyclic substituent or of a long side chain makes the substrate more susceptible to attack. The N-methyl derivatives of the basic or acidic amino acids are only slowly acted upon. Some preliminary evidence points to the presence of a flavin as the prosthetic group of the demethylase.

The cleavage of the benzene ring by bacterial systems has become a subject of much attention. Stanier and his group (325 to 327) have elucidated the enzymatic mechanism by which protocatechuic acid (3,4-dihydroxybenzoic acid) is converted to β-ketoadipic acid ($COOHCH_2COCH_2CH_2COOH$) in extracts of *Pseudomonas fluorescens*. One or more oxidases catalyze the oxidation of protocatechuic acid to β-carboxy muconic acid ($COOHCH{=}C(COOH)CH{=}CHCOOH$), with uptake of two atoms of oxygen. The attack is by way of the —COH—COH—

linkage in the ring. Oxidation ruptures the bond between the two ring carbons and two carboxyl groups are formed. The sequence of events in the decarboxylative conversion of β-carboxymuconic acid to β-ketoadipic acid, the final product, is still unknown. However, Sistrom & Stanier (327) have shown that the analogous enzymatic conversion of *cis-cis*-muconic acid to β-ketoadipic acid proceeds via the lactone, (+)-γ-carboxymethyl-Δ^{α}-butenolide. The *cis-trans*-isomer of muconic acid is much more slowly converted by the same enzyme to the (−)-lactone. This is an interesting case of an enzyme which can produce two different optical isomers depending upon the geometrical configuration of the substrate. Finally a delactonizing enzyme intervenes which converts the butenolide to β-ketoadipic acid.

Luminescence[17]

The *in vitro* reconstruction of an enzyme system which produces light during oxidation has been the objective of Newton Harvey and his school over many years of investigation. This objective is now close to being attained. When luminous bacteria are precipitated with acetone an aqueous suspension of the "acetone" powder emits such minimal luminescence when shaken with oxygen that it can be detected only by measurement with ultrasensitive light-detecting instruments. Strehler (237) and McElroy *et al.* (238) independently have discovered the requirement of both DPNH and FMN for production of brighter luminescence by these bacterial extracts. Now Strehler & Cormier (329) have identified in palmitic aldehyde a third factor which leads to a 100- to 200-fold increase in the luminescence in presence of excess DPNH, FMN, and molecular oxygen. Although this factor has been isolated from hog kidney cortex powder it is a reasonable presumption that a similar substance in luminous bacteria plays the same role in the luminescent reaction. The details of the enzymatic processes in which DPNH, FMN, and palmitic aldehyde participate are less clear than their requirement. In a general way it can be stated that FMN is reduced by DPNH in presence of some dehydrogenase. Whether FMN is free or bound is still undetermined. Then $FMNH_2$ undergoes oxidation by molecular oxygen. Strehler & Cormier (329) assume that palmitic aldehyde is the cofactor for this oxidative reaction and that in the course of this oxidation $FMNH_2$ is oxidized to FMN in an excited state which emits light and then reverts to the ground state.

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NONOXIDATIVE, NONPROTEOLYTIC ENZYMES[1,2]

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Despite a well-intentioned author the subject matter covered by the above title does not lend itself well to a critical review as contrasted to a somewhat vapid catalogue. Those enzymes which find their way into this chapter do so by default. There are many nonoxidative and nonproteolytic enzymes but as soon as their functions in the metabolic schemes of biology become known they are snatched away to join their partners in important and intriguing nexi of enzymes in another chapter. This is as it should be, and it is to be hoped that the isolated and forelorn enzymes which populate the bulk of this chapter will not be too meanly regarded; they too may sometime find an honorable and useful place. The literature covered in this review comprehends, more or less, the period of December, 1953 to November, 1954, with occasional excursions into the more distant past for some pertinent references not previously discussed. It is, of course, impossible to pretend a complete coverage. Omissions have often been made on the basis that some enzymes will be discussed under another cateogry in this or succeeding volumes. Space limitations have necessitated an almost ruthless selection, sometimes arbitrary, and undeniably influenced by the interest of the writer. Where two or three papers by the same authors deal with phases of the same subject matter, usually the most complete and not necessarily the first has been cited in the bibliography.

Phosphatases

Nonspecific monoesterases.—Although phosphatases have been more intensively studied than any other class of enzymes no nonspecific phosphatase has yet been crystallized. Hence the claim of Helferich *et al.* (1) that they have succeeded with potato acid phosphatase warrants special attention. These workers, on adding ethanol to a solution of purified phosphatase in dilute ammonium sulfate obtained a precipitate containing a mixture of ammonium sulfate crystals with other crystalline material which they suggested might be phosphatase. However the latter material could not be recrystallized. Helferich & von Bruck (2) subsequently improved their purification procedure to obtain a preparation more than twice as active as the crystals, but the latter preparation has not been reported as crystalline. The final product, 120,000 times as active per unit weight as the starting material, still

[1] The author is greatly indebted to Professor A. K. Balls of this University for much helpful discussion and advice during the preparation of this review.

[2] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; INH for isonicotinic acid hydrazide; UDP for uridine diphosphate; UTP for uridine triphosphate.

contained 14 per cent carbohydrate. From the data given it is calculated that their best preparation had a turnover number of 45,000 moles per min. per 100,000 gm. of enzyme. This may be compared with the prostatic acid phosphatase purified by London & Hudson (3) which has a turnover number of 100,000. Although both groups of workers used β-glycerophosphate the prostatic phosphatase was assayed at 37°C. with a substrate concentration of 0.5 per cent (which is considerably less than saturating) whereas the potato enzyme was assayed with 1.5 per cent glycerophosphate but at a temperature of 30°C.

Mathies (4) has purified swine kidney alkaline phosphatase to the point that it has a turnover number of over 100,000 moles per min. per 100,000 gm. of protein with phenyl phosphate at 25°C. Studies made with highly purified preparations indicate that the so-called activating effect of amino acids is shared by a number of other substances capable of forming complexes with metals and is attributable to the removal of trace metals. Cu^{++} is reported an efficient inhibitor of the spontaneous reactivation of this enzyme.

Morton (5) has now reported in detail the application of butanol to release alkaline phosphatase from the insoluble particles with which it is associated in homogenates of calf intestinal mucosa. The activity is largely to be found in the microsomes. It has been calculated that his best preparation has a turnover number of about 45,000 if a M of 100,000 is assumed for his enzyme.

Buroughs (6) has presented support for the presence of "isodynamic" acid phosphatases in the cytoplasmic phosphatases of sugar beet, spinach, and tobacco leaves, on the basis of acid dialysis and treatment with ion exchange resin. The existance of more than one phosphatase was also indicated by immunological experiments. Thus sugar beet phosphatase could be separated into two fractions with different K_M values for *p*-nitrophenyl phosphate. Preparations subjected to acid dialysis underwent inactivation which could be partially reversed by Co^{++}, Cu^{++}, Fe^{++}, and Mn^{++}. Cu^{++} was the most effective. Maximum activity was obtained at 5×10^{-4} *M*, higher concentrations causing inhibition. It would be of value in studies of this sort to establish whether or not the so-called isodynamic enzymes show any difference in specificity for a number of diverse substrates. It must be confessed that the term "nonspecific" is a cover for the general lack of knowledge concerning the properties and function of these enzymes.

Schlamowitz (7) has postulated the existence of two different phosphatases in purified preparations of dog intestinal mucosal phosphatase on the basis of its reaction against its antiserum. Whereas all of the activity was precipitated in the antigen-antibody reaction as normally carried out, the presence of substrate prevented 12 per cent of the phosphatase from precipitating. Schlamowitz has concluded that the antibodies to the minor component are directed to an enzyme site, whereas the major component is attached to its antibody at some nonactive site. This worker (8) also found that in contrast to the complete precipitation of dog intestinal phosphatase by the

antiserum (in the absence of substrate) there was no precipitation of dog liver and kidney phosphatase, nor intestinal phosphatases from rat, rabbit, and bovine sources. Precautions were taken to insure that negative results were not a result of excess of antigen. All of these preparations were similar with respect to the effect of pH, Mg^{++}, cyanide, fluoride, and histidine; the only observed differences were immunological.

Naidoo & Pratt (9) have reported that thiamine pyrophosphatase and glycerophosphatase, both of the rat cerebral cortex, are distinct entities, on the basis of differential inhibition and inactivation of these two activities with acetone and alcohol in histological tests.

Thionophosphatase.—Neuberg who has discovered so many of the substrates susceptible to phosphatases has, in conjunction with Forrest & Grauer (10), added still another: monophenylthionophosphate ($S=P(OH)_2OC_6H_5$). "Thionophosphatase" which occurs in *Aspergillus oryzae* may also possibly attack the diphenyl compound, albeit slowly, but does not hydrolyze the triphenyl derivative.

Induction of phosphatase by light.—The effect of light on the content of phosphatase in the leaves of *Kalenchoë blossfeldiana* has been reported by Ehrenberg (11). At the end of the dark period in a 12 hr. light–12 hr. dark cycle the enzyme is at its lowest ebb. The concept that there can be a cyclic variation of enzyme content in higher plants in such a short time is of fundamental importance, and it is to be hoped that this phenomenon will be further examined.

Phytase.—Inositol monophosphate obtained by enzymatic degradation of phytic acid has been shown by Fleury *et al.* (12) to be identical with synthetic 2-phosphoinositol [nomenclature of Fletcher *et al.* (13)] by means of x-ray powder photography and to be different from the monophosphate yielded on the alkaline hydrolysis of phytic acid.

Steenbock *et al.* (14) have reported that vitamin D, which is known to improve the utilization of phytate phosphorus, causes an increase in intestinal phytase when administered to chicks and rats on a cereal rachitogenic diet. Hill (15) has made a fairly critical study of the various factors which effect cereal phytase activity *in vitro*. He has confirmed the high activity in wheat but finds none in oats.

Hexose phosphatase.—According to Kuhlman (16) the free fructose which occurs in seminal fluid is produced in the reproductive glands of the male and most probably arises from the action of specific hexose phosphatases which act on glucose-6-phosphate and fructose-6-phosphate. The latter compound is more readily split. No effort was made to exclude the possibility of phosphohexose isomerase action.

Fructose-1,6-diphosphatase.—Pogell & McGilvery (17) have obtained a preparation of fructose-1,6-diphosphatase with a specific activity some three hundred times greater than that of the rabbit livers from which it was prepared. The specificity of this enzyme has borne out the expectation of a number of workers in the field who have used less purified preparations. The

enzyme is specific for the phosphate in the 1-position of fructose. The 6-phosphate group in 1,6-sorbose diphosphate is structurally analogous to the 1-phosphate group of fructose diphosphate, but if this group is attacked by the enzyme at all, it is only attacked feebly.

Inhibitors.—Ehrenberg (18) has noted that erroneous information regarding the enzyme content of certain leaves can result from the inhibitory action of the tannins present. Thus she found low results for phosphatase and amylase in the leaves of *K. blossfeldeana.* The inhibition could be partially relieved by the use of powdered hide. It is interesting that Helferich and co-workers (1, 2) used differential precipitation with tannin in purifying potato phosphatase.

The well-recognized inhibitory action of molybdate on acid phosphatase has been studied by Spencer (19) with crude tomato extracts. Because of the low value of the competitive inhibition constant ($K_I = 10^{-5}$ *M*) and the ability of molybdate to cause inhibition of phosphatase within intact root tissue, Spencer has suggested that molybdate in physiological concentrations may act *in situ* as an inhibitor and that the high content of inorganic phosphate in molybdenum-deficient plants may reflect a less inhibited phosphatase system. Considering the extremely low concentrations of Mo which suffice for most plants, one would have to postulate a very high localization of Mo indeed to produce this effect, despite its powerful inhibiting action.

Vantaggi-Cozzari & Levi (20) have stated that 2,4-dinitrophenol inhibits alkaline phosphatase whereas neither the 2,5- nor the 2,6-dinitrophenols do so. The 2,6- and 1,6-dinitro-1-naphthols are also inactive.

Enzymes attacking phosphorylated esterase inhibitors.—Augustinsson (21) has found an enzyme which inactivates N-dimethylamineethoxycyanophosphate. He has assigned the name, "tabunase" thereto, after the trade name of its substrate "Tabun." Specificity studies with an admittedly impure enzyme strongly support the view that all phosphoacyl cholinesterase inhibitors act as substrates. The chloro compound is split more rapidly than "Tabun" itself while the methoxy derivative is more readily attacked than the higher alkoxy homologues. Tetraethylpyrophosphate and diisopropylfluorophosphate are also hydrolyzed. Plasma cholinesterase in combination with "Tabun" is reactivated by tabunase. Augustinsson & Heimbürger (22) find the enzyme in the plasma of man and a number of other mammals as well as in various other tissues. The parallel distribution of tabunase and the diisopropylfluorophosphatase of Mazur (23) makes it likely that these activities are the properties of the same enzyme. Indeed Augustinsson & Heimbürger (22) have proposed the more general name phosphorylphosphatase for this enzyme, unfortunately imputing a broader specificity than was intended. On the other hand the behavior of plasma and kidney preparations suggests the occurrence of two different phosphorylphosphatases or tabunases. The authors (24) have shown that the hydrolytic products, HCN and the cyanide-free diester, are inhibitory to tabunase thus explaining the failure of the enzyme to completely inactivate "Tabun." They postulate that the

action of "Tabun" on cholinesterase occurs by displacement of the cyano group by the enzyme molecule which is attracted by its electronegative center to the positively polarized phosphorus atom of the inhibitor. The cholinesterase, now inactive by virtue of being phosphorylated, may then be regarded as a close analogue of the original "Tabun," (wherein the cyano group is replaced by the enzyme) which is also susceptible to tabunase action, and hence reactivatable. These workers suggest that inasmuch as the enzyme does not cleave N-P bonds, its ability to reactivate inhibited cholinesterase implies that the inhibitor is not attached through a nitrogen atom but possibly through a serine hydroxyl or through one of the phenolic groups of an unspecified diphenol. However, since the specificity of the enzyme with respect to such bonds in general has not been examined, this observation ought not to be considered of crucial importance in deciding where the phosphate is attached to the inhibited enzyme. These workers (25) found that neither acetylcholine, choline, physostigmine, or prostigmine affected the hydrolysis of "Tabun" and diisopropylfluorophosphate by rabbit plasma. None of several "ordinary" phosphatases or esterases attacked "Tabun" or diisopropylfluorophosphate.

Mounter & Chanutin (26) having previously shown that Mn^{++} or Co^{++} and certain specific factors are required for full activity of hog kidney diisopropylfluorophosphatase, find that in the presence of Mn^{++} only certain imidazole and pyridine derivatives are effective and that these actually prevent the activating effect of Co^{++}. Proline and hydroxyproline, on the other hand are effective with Co^{++}. The metal and cofactor should be present in equivalent concentrations; an excess of cofactor causes inhibition.

Activators of phosphatase.—The question of the existence of coenzymes for phosphatases is one of long standing. Besides the difficulty in showing an absolute requirement coupled with the lack of a pure enzyme preparation with which to demonstrate it, there has been the odd nonspecific stimulatory effect of numerous amino acids that has been intensively studied by Bodansky (27) [but see Mathies (4)]. Hence it is of particular interest that Lora-Tamayo & Alvarez (28) claim uridylic acid to be a component of kidney phosphatase. They introduced C^{14}-labeled orotic acid (a well established precursor of uridylic acid) into rabbits and rats. They obtained from the kidneys of the animals so treated, a radioactive phosphatase preparation whose specific radioactivity increased with purification. The radioactive moiety was released into solution by trichloroacetic acid and behaved on paper chromatography as uridylic acid. Unfortunately, no data are given which permit the calculation of the molar proportion between uridylic acid and enzyme or protein. The approach of these workers is especially well suited to give an answer to this question, and a more detailed announcement is awaited with interest.

Phosphatase "apoenzyme" from pig kidney prepared by dialysis against borate and carbonate according to Kumai (29) has been further studied by Akamatsu & Kobayashi (30) from the point of view of its coenzyme require-

ment. "Coenzyme" could be obtained in the supernatant liquid of heated enzyme solutions prepared from kidney, rice bran, or human urine. Histidine caused activation. Racemic, *d*- or *l*-histamine was somewhat less active than histidine while other amino acids were in general about 1/20 to 1/30 as effective. It should be noted that the "apoenzyme" showed, without the addition of any activator, an activity about 10 per cent of that obtained on maximal activation. Benzoylhistidine and histidine anhydride inhibited this residual activity [Akamastu & Aso (31)]. These workers (32) also found, in addition to the substances noted above that various peptides including carnosine were activators, and that even casein had some effect. Preincubation of the apoenzyme with the peptide was required for maximum effect, although no hydrolysis of the peptide occurred. About 50 per cent of the maximum activity could be realized in the absence of Mg^{++}.

The absolute requirement of rat erthrocyte pyrophosphatase for Mg^{++} is attributable according to Bloch-Frankenthal (33) to the fact that the complex $(MgP_2O_7)^{=}$ is the actual substrate of the enzyme.

Nucleotide phosphatases.—Most of the interesting developments in this field have been concerned with characterizing the action of the nucleases, thus permitting their use in the determination of the structure of nucleic acids and their component nucleotides (cf. chapter on Nucleic Acids, Purines, and Pyrimidines).

Wang (34) reports that soil bacterium "U-1" is another source of 5′-nucleotidase. It attacks 5′-adenylic and 5′-inosinic acids and, as expected, is inactive against a number of 2′ and 3′ nucleotides. The enzyme does not act on 2′,5′-diphosphoadenosine or 3′,5′-diphosphoadenosine. This suggests that the 2′ and 3′ hydroxyl groups are essential to the activity.

The development of 5-adenylic acid phosphatase in the maturing rat cerebral cortex has been studied by Naidoo & Pratt (35). During maturation the increase in activity is most rapid between 15 to 80 days after birth. The activity seems to arise first in the nerve fibers (in which no activity is apparent for the first few days). They suggest that this enzyme may be concerned with myelination.

McDonald & Kaufmann (36) have found that a number of proteins, especially basic ones, such as lysozyme and cytochrome-*c*, can simulate the action of ribonuclease on apurinic acid.

Myosin and adenosinetriphosphatase.—Mommaerts & Green (37) have clarified the apparent double pH optimum of the adenosinetriphosphatase activity of myosin. Purified myosin shows a sharp optimum at pH 6.4 which is accentuated by high concentrations of Ca^{++}. Aging abolished the increase attributable to added Ca^{++}. The apparent alkaline optimum that has been hitherto observed is the resultant of an increasing rate of hydrolysis and an increasing rate of enzyme inactivation with increasing hydroxyl ion concentration. There is no alkaline optimum, the activity being proportional to the hydroxyl ion concentration when Ca^{++} concentration is optimal.

The cleavage of crystalline meromysin by trypsin into a light and heavy

fraction as first done by Szent-Györgyi (38) has been repeated by Nekhorocheff *et al.* (39) who took precautions to avoid undue inactivation of 5-adenylic acid deaminase. They verify that the adenosinetriphosphatase is associated with the heavy fraction and find the deaminase largely but not completely in the light fraction.

The role of divalent metals in adenosinetriphosphatase activity of myosin, always a subject of some uncertainty, has for the time being become further complicated by Friess' (40) remarkable finding that ethylenediaminetetraacetate causes an acceleration in dephosphorylation. The effect begins at concentrations of 10^{-4} *M* and reaches a maximum at 10^{-2} *M*, but if Ca^{++} is present, the chelating agent does not begin to exert its action until its concentration exceeds that of the Ca^{++}. Myosin which has once been exposed to ethylenediaminetetraacetate and then dialyzed loses its sensitivity to Ca^{++}, although it still responds to the chelating reagent. Perhaps the loss of effect of Ca^{++} can be linked to the extra aging which the enzyme undergoes during the preliminary treatment and dialysis [see Mommaerts & Green (37)]. On the other hand, Gross (41) found that ethylenediaminetetraacetate, under various conditions, could accelerate or inhibit the hydrolysis of ATP^2 by mouse heart homogenate. He explained this by assuming that the reagent removed inhibitor to cause acceleration and that it combined with Ca^{++} to produce inhibition.

The idea that the activity of animals and the level of adenosinetriphosphatase content of their muscles run parallel has met support in several instances. According to Davison & Richards (42), the quantitative level of muscle apyrase activity in the cockroach, crayfish, and minnow at low temperature is of the same order as the activity of the intact animal, itself, in nature.

The physical activity of the spermatazoal tail is suggestive of the contractile power of muscle fibre and the adenosinetriphosphatase associated with myosin. Nelson (43) has indeed found that the predominant quantity of this activity in the spermatazoon is localized in the tail.

The anomalies associated with the ATP-hydrolyzing enzymes encountered in mitochondrial studies are illustrated by the behavior of the apyrase of mammary gland (lactating rat) homogenates. Craine & Hansen (44) have found that Mg^{++} depresses the breakdown of ATP in homogenates in phosphate buffer but stimulates it when H_2O is the suspending medium. Water extraction of the mitochondria removed about half of the enzyme that was stimulated by Ca^{++} or Mg^{++}. Both the soluble and insoluble fractions had true apyrase activity as they removed at least two of the phosphate groups of ATP.

The presence of more than one enzyme attacking ATP in toad embryo homogenate is suggested by Maruyama (45) who finds that dinitrophenol causes an increase in apyrase activity while azide abolishes this increase but has no effect on the original (unstimulated)activity.

Diesterases.—Akamatsu & Nishibori (46) have found that "Habu"

venom contains a diesterase which possesses "phosphotransferase" activity as well as the usual hydrolytic activity. Thus ethyl *p*-nitrophenyl phosphate in the presence of the enzyme yields ethanol and *p*-nitrophenol phosphate. However if an acceptor alcohol is also present, some diester phosphate is formed in addition. Such a transfer was demonstrated by adding urinary phosphomonesterase to the reaction products. When no transfer occurred, an amount of inorganic phosphate equivalent to the nitrophenol was liberated; when, however, transfer took place, the difference in equivalents between free nitrophenol and inorganic phosphate was equal to the amount transferred. Glycol and glycerol both served as acceptors. The reaction with a pig kidney diesterase which also had transferase activity was complicated in an interesting manner because this preparation, although devoid of monoesterase activity could hydrolyze either the nitrophenyl or the ethyl group of the original substrate. Diesterases prepared from rat or ox liver and kidney did not demonstrably catalyze transfer.

Snake venom diesterase has been used by Perlmann (47) to show the nature of the phosphate grouping in β-casein. She had demonstrated earlier that phosphomonoester groups were absent by the inertness of the phosphate groups to prostatic phosphomonesterase. However, following the action of diesterase from *Crotalus adamanteus*, prostatic phosphatase liberated 72 per cent of the phosphate as well as some small peptides, thus suggesting the functioning of the phosphate groups as bridges between peptides. The same technique also revealed that the phosphate group in pepsin occurs as a diester.

Akamatsu & Kato (48) have determined the K_M values for diesterase with diphenyl phosphate and *p*-nitrophenyl phosphate as well as the inhibitor constants for a number of phosphate compounds. Phenylphosphate, inorganic phosphate, *p*-nitrophenylphosphate, methylphosphate, and pyrophosphate were competitive inhibitors in increasing order. Diethylmethylphosphate was without effect.

Phosphoprotein phosphatase.—Sundararajan & Sarma (49) have obtained a 200-fold purification of phosphoprotein phosphatase from ox spleen. This preparation was, however, not entirely free of monoesterase activity. They did, however, succeed in preparing the enzyme from rat spleen with no detectable activity against β-glycerophosphate. The rat enzyme, very similar to that of the ox, has a pH optimum of 5.8, is activated by thioglycolic acid, attacks both α- and β-casein, phosvitin and vitellin but not phosphopeptone. Except for the last case it strongly resembles the stomach mucosal enzyme already described by Mattenheimer (50).

Physiological role of phosphatase.—A summary of some of the evidence bearing on the role of phosphatase in bone formation has appeared in an article by King (51) which deals with plasma alkaline phosphatase in disease.

A possible role for cell-membrane phosphatase is suggested by the experiments of Volk (52) in which it is shown that *Propionibacterium pentoaceticus* which can grow on either free sugar or phosphorylated sugar under normal conditions, can utilize only the free sugar when grown on fluoride.

Phosphate Transferring Enzymes

Polyphosphate kinases.—Hoffmann-Ostenhof *et al.* (53) have reported a new phosphate-transferring enzyme in yeast which deserves special attention because it may possibly provide a means for the utilization of the "high energy" of the metaphosphate by reactions involving the polyphosphonucleotides. The enzyme, which catalyzes the transfer between metaphosphate and ADP,[2] is nonextractable, has a pH optimum of 6.7, and is fluoride sensitive. The reaction has so far been shown to occur only in the direction stated above, using P^{32}-labeled metaphosphate. Hoffmann-Ostenhof (54), employing the rational system of nomenclature which he proposed earlier, has named the enzyme "metaphosphate→ADP-transphosphorylase," but he has made a concession to tradition and conversation by also offering the alternative "metaphosphate kinase." In connection with this discovery it may well be that Yoshida's (55) observations are directly concerned with the same enzyme. This worker obtained a cell-free system from phosphorus-starved yeast which catalyzed the formation of metaphosphate from inorganic phosphate provided a glycolytic substrate or ATP was present. What may be a related type of activity has been found in yeast autolyzate by Kornberg & Kornberg (56). In this case the reaction of ATP with pyrophosphate produces a linear triphosphate.

Metaphosphate is being increasingly encountered in the lower plants. The very reasonable idea that metaphosphate serves these organisms as a reserve of phosphate and of energy for later proliferation does not appear to have any application to the rather amazing discovery of Winder & Denneny (57) that tetrahydrofurfuryl alcohol stimulates the production of metaphosphate in rapidly growing cultures of *Mycobacterium tuberculosis* and *smegmatis*, which would normally be using up their metaphosphate reserve.

ATP-creatine transphosphorylase.—A very complete description of the preparation and assay of crystalline ATP-creatine transphosphorylase has been made by Kuby, Noda & Lardy (58, 59). The preparation based on alcohol precipitation in the presence of Mg^{++} has given yields of crystalline enzyme as high as 80 per cent of the original content of rabbit skeletal muscles. The crystalline material is homogeneous by a number of criteria and has a M of 81,000. An examination of the kinetics (60, 61) showed that ATP and ADP participate in the reaction as their Mg^{++} complexes. The forward reaction and the reverse reaction have pH optima of 9 and 6 to 7, respectively, with higher pH values leading to greater final concentrations of creatine phosphate.

Nucleotide-transferase.—The observation that calf liver acetone powder contained an enzyme which dephosphorylated UDP[2] far more rapidly than UTP,[2] ATP, and ADP led Strominger, Heppel & Maxwell (62) to the discovery that the enzyme was apparently specific for UDP and the reason the other substrates were effective was the presence of a nucleotide monophosphate kinase and traces of UDP, ATP, and myokinase. The enzyme (which may be a mixture of two enzymes) catalyzes the following reaction:

$$\text{ATP} + \text{uridine monophosphate} \rightleftarrows \text{ADP} + \text{uridine diphosphate}$$
$$\text{AMP} + \text{uridine triphosphate} \rightleftarrows \text{ADP} + \text{uridine diphosphate}$$

Cytidine triphosphate was also active in this system. It will be noted that the kinase is actually a sort of a mixed myokinase. A new member of the "nudiki" (nucleoside diphosphokinase) group of enzymes has been discovered by Sanadi *et al.* (63) in a heart muscle preparation catalyzing the following reaction:

$$\text{succinyl-CoA} + PO_4^{=} + \text{ADP} \rightleftarrows \text{succinate} + \text{CoA} + \text{ATP}.$$

This reaction (ostensibly phosphorylating ADP to give ATP) was found to depend for its operation on an impurity in the ADP or CoA.[3] Further examination made it apparent that guanosine diphosphate was the primary acceptor and the formation of ATP was attributable to the "nudiki"-catalyzed reaction:

$$\text{Guanosine triphosphate} + \text{ADP} \rightleftarrows \text{guanosine diphosphate} + \text{ATP}$$

Inosine nucleotides were also operative in the succinyl-CoA connected phosphorylation.

Transfer induced by light.—Takashima & Mitsui (64) have noted that the amount of phosphate which spinach leaf homogenates liberate from α- or β-glycerophosphate is greater in the dark than in the light. They have reported the presence of an unidentified labile-phosphate compound and interpret their finding as evidence that light induces a direct transfer of phosphate from the substrate to some acceptor. It is, of course, not impossible that they are observing the usual light-coupled oxidative phosphorylation which consumes some of the inorganic phosphate arising from the action of phosphatase on the substrate.

Carboxylic Acid Esterases

Lipase.—A most interesting investigation of the optical specificity of seed lipases has been reported by Bamann *et al.* (65). Although none of the preparations show an absolute specificity, there is a preference for one of the optical isomers of methylmandelate by the crude preparations. Thus *Chelidonium majus* and *Nigella damascus* hydrolyze the (+) ester more rapidly, while *Nigella sativa*, a member of the latter genus, prefers the (−) ester, as does *Ricinus communis*. What is more remarkable is that the optical specificity of the water insoluble enzyme differs from that of the soluble enzyme from a given species. Differences in optical specificity of enzyme extracts from the same seed meal can arise as a result of the extraction time because the ratio of desmo and lyo enzymes changes. It makes a difference whether a fixed amount of hydrolysis by a given quantity of seed meal is allowed to occur quickly (by providing excess substrate) or slowly (by using low concentrations of substrate). Even the addition of a few drops of toluene to 50 ml. of reaction mixture can alter the specificity of the hydrolysis.

Hofstee (66) has shown that the linear plot of the Michaelis equation, in

which V is plotted against V/s is a sensitive means for revealing deviations from theory, especially at low substrate concentrations. He has applied this treatment to an analysis of the action of horse liver esterase on a series of aliphatic carboxylic acid esters of *m*-hydroxybenzoic acid (67). He observed two discontinuities with the ester of C_7 acid. The first, which occurred at the higher substrate concentration, was attributed to micellar formation. The second was stated to be a consequence of the enzyme structure, which was not otherwise explained. An interesting and novel manner of stopping the reaction was by the addition of parathion. Hofstee (67a) also found that in the homologous series of esters made with C_3 to C_{12} acids the K_M values were not particularly effected, but the catalytic efficiency as given by V_{Max}/K_M increased exponentially by a factor of about 2 to 2.5 for each upward step in the series.

Borgström (68) has studied the pH optimum of fat digestion with rat pancreatic lipase, simulating *in vivo* conditions. The addition of taurocholic acid shifts the pH optimum from 8 to 6 to 7, which is the range found in the upper portion of the rat intestine. He has also found that in the hydrolysis of long chain fatty acid triglycerides by rat pancreatic juice an exchange occurs between the acids in the 1 and 3 positions and free acids (69). Part of the exchange is a result of real synthesis. The short chain fatty acids are not as readily incorporated into glycerides as the longer ones. The 1,2 diglyceride is preferentially formed on hydrolysis.

Gawron *et al.* (70) have studied the effect of substituents on the hydrolysis of phenylacetate by wheat germ lipase. The results obtained with substituents of varying electronegativity is not in disagreement with their idea that the induced variation in the electrophilic character of the carboxyl carbon of the substrate can influence the strength of the enzyme substrate complex and the rate of hydrolysis. They have presented a simple exposition of what is commonly regarded as a reasonable mechanism.

Esterases.—A most welcome and lucid discussion of the properties of esterases and their distinction from lipases and including experimental data bearing on this matter has been presented by Aldrige (71).

The preparation of a number of alkyl naphthyl carbonates as chromogenic substrates for esterases is described by Tsou & Seligman (72). The use of similar substrates as well as chromogenic acetates and caprylates for differentiation between "lipase" and "ali esterase" has also been described [Ravin & Seligman (73)].

The hydrolysis of methyl and ethyl thioacetates by various minced tissues of rats was shown by Suzuoki-Zirô & Suzuoki-Tuneko (74) to proceed more rapidly than that of the respective acetates. With acetone liver powders these activities remained parallel throughout fractionation and were therefore presumed to reside in the same enzyme. Both activities were inhibited by 0.0001 *M* atoxyl.

By the ingenious device of supplying substrate in toluene to an aqueous solution of the enzyme, Bergmann *et al.* (75) have been able to demonstrate

the hydrolysis of carboxylic acid anhydrides with a liver esterase and cholinesterase. Dibenzylacetate phosphate was inhibitory. They were also able to demonstrate transfer of an acyl group from an anhydride to choline (76).

Lecithinase.—Hanahan, Rodbell & Turner (77) have tested the lecithinase A of *Naia naia* and *Crotalus adamanteus* venoms on synthetic dipalmitylecithin and dipalmitoyllecithin. In both instances, one acyl group is removed leaving a lysolecithin. Hanahan (78) has shown that with the enzymes from the above mentioned snake venoms or from pancreas the resulting lysolecithin is free in the 1 position.

The so-called lecithinase D which splits choline from lecithin has been shown to be localized in the plastids of sugar beet, spinach, and cabbage leaves and carrot root by Kates (79). He had previously shown that the presence of diethylether was required for the reaction (80).

Hanahan & Vercamer (81) have employed *Clostridium perfringens* type A toxin on synthetic dipalmitoleyl-L-α-lecithin and dipalmitoyl-L-α-lecithin to give, in the presence of 98 per cent diethyl ether—2 per cent ethanol, a yield in excess of 90 per cent of phosphorylcholine and the corresponding 1,2 diglyceride.

Hayaishi & Kornberg (82) have obtained partially purified preparations from *Serratia plymuthicum* which can form glycerophosphocholine from lecithin and lysolecithin. The lysolecithinase activity of the cells could be increased by 14-fold by growing them on lecithin. An enzyme called glycerophosphocholine diesterase which could form choline and α-glycerophosphate from the deacylated lecithin was partially purified. The enzyme had no effect on diphenylphosphate or polynucleotides. It is probable that the same enzyme also splits phosphoglyceroethanolamine. The enzyme was strongly inhibited at low concentrations of Mg^{++}, Mn^{++}, or Zn^{++}, and also by small amounts of ethylenediaminetetraacetate. No metals which were tested overcame this inhibition.

The use of lecithinase A as an aid in elucidating the structure of mitochondria has been further extended by Nygaard, Dianzani & Bahr (83). Thus the previously observed disturbance of mitochondrial activity by lecithinase A has been shown to be essentially reproducible by lysolecithin. Not only is the rat mitochondrion disintegrated, but the succinoxidase system is inactivated. While this does not necessarily prove that the phospholipide is essential to the mitochondrion it does establish that the partially deacylated product is harmful. Neither lecithinase C (which can split off phosphocholine from the phospholipide) nor trypsin had any effect. The negative result with lecithinase C should not imply that the lecithin is unessential to mitochondrial integrity unless it is further established that lecithinase C really did attack the mitochondrial lecithin.

Glutathione thiolesterase.—This enzyme has been reported by Kielly & Bradley (84) to be in mouse liver extracts. It acts on S-acetyl and S-butyryl glutathione and because of the changing K_M ratios noted on purification it may be two enzymes. It is definitely not identical with liver esterase nor with glyoxylase II.

Inhibitors.—A flood of publications continues in the field of esterase and cholinesterase inhibitors of which only a few can be mentioned: Wilson (85) has investigated the effect of alkyl substitution on the inhibitory power of ethanolamine and discussed the theoretical implications. Friess & McCarville (86) have compared various reversible inhibitors of cholinesterase in support of their theory that a potent inhibitor must have a region of high electron density separated by a distance of one methylene group from a polymethylated N atom.

Klupp & Stumpf (87) have explained the greater sensitivity of the dog than the human to choline diesters of dicarboxylic acids by showing that these compounds are much more slowly cleaved by dog serum.

Among a number of dialkyl phosphoric esters of hydroxypyridines and hydroxyquinolines tested by Andrews *et al.* (88) as anti-cholinesterases, 3-(diethoxyphosphinyloxy)-N-methylquinoliniummethyl sulfate has the highest activity *in vitro* yet recorded for a phosphorus compound. This compound caused 50 per cent inhibition of erythrocyte cholinesterase in a concentration of 1.5×10^{-10} *M* and had a LD_{50} in mice of 0.023 mg. per kg. (intravenous administration). An imposing number of cholinesterase inhibitors have been made and tested by Kolbezen *et al.* (89) who present a good theoretical discussion of the effects of structure on activity.

Serine phosphoric acid has been isolated in 40 per cent yield from diisopropylfluorophosphate-inhibited cholinesterase by Schaffer *et al.* (90), but whether this compound is formed by migration of the phosphoryl group during hydrolysis is still an open question just as in the case of chymotrypsin.

Cohen *et al.* (91) have obtained significant inhibition of esterase in extracts of various species of *Mycobacterium* with polymixin B. Inhibition was more pronounced with the highly insoluble substrate β-naphthyllaurate than with tributyrin or monobutyrin. No inhibition was observed with esterase from *Pseudomonas aeruginosa*, rabbit serum, or bovine liver. Cationic detergents were also effective inhibitors. The authors have ascribed the anti-esterase activity to the detergent-like action of the antibiotic.

A possible explanation for the toxicity of γ-hexachlorocyclohexane is suggested by Keller's (92) finding that liver and brain esterases of mammalian origin as well as those from house flies are stimulated by this compound. The α- and β-isomers were without effect, and the response of the enzyme from resistant flies was less than that from normal flies. The differences were not great and the mode of action of this insecticide cannot be regarded as definitely established.

Glycosidases

It is not planned to review glycosidases in this chapter, but several developments in this field that are somewhat remote from the mainstreams of major metabolic systems warrant mention.

Cellulase.—Rather interesting for its ecological implications is the observation by Hirsch (93) that *Neurospora crassa* grown on glycerol in the presence of filter paper produces extracellular cellulase if grown at 35°C., but

not at 25°C. This is an instance of induced enzyme formation. If amino nitrogen is present, no cellulase is formed. The rampant growth of *N. crassa* in tropical or subtropical ranges in the wild state may be explained by its ability to elaborate cellulase.

Chitinase.—Hackman (94) has studied the action of chitinase on the chitin of lobster cuticle and fly puparia and on chitin sulfate. The only hydrolytic products detected were D-glucosamine and acetylglucosamine. Both chitin and chitin sulfate were optimally degraded at pH 4.8. It is not possible to state specifically what the source of the enzyme was, inasmuch as the intestinal tract of the snail together with its contents was used.

Enzymes acting on polygalacturonic acid.—Well authenticated cases of increase in enzyme content in response to hormone treatment are of considerable interest. Bryan & Newcomb (95) have found that the pectinmethylesterase activity of tissue cultures of tobacco pith increases to twice that of the controls when indoleacetic acid is given. Pectin, which is believed to be, at least in part, responsible for the structural rigidity of the cell wall, must first be demethylated before it can undergo degradation by polygalacturonidase. The enzyme activity is localized on the solid fraction of the homogenate arising from the cell wall. The fact that the intact cell is active against the nonpenetrating pectin molecule is taken as evidence that the enzyme is localized on the surface. The hormone has no effect on the homogenate. The authors have emphasized the significance of their findings to the "plasticizing" theory of indoleacetic acid action. According to this theory the softening of the cell walls accounts for the elongation effects of this plant hormone.

The end-products of polygalacturonase action have been the objects of investigation in several quarters. Jones & Reid (96) isolated the mono-, di-, and trigalacturonic acids from the action of the "D" type enzyme of *Aspergillus foetidus*. The digalacturonide was further hydrolyzed by the "S"-type enzyme. Demain & Phaff (97) utilizing a yeast enzyme found cleavage to cease at 70 per cent over-all hydrolysis. Digalacturonic acid was not attacked. The rate of hydrolysis of the longer-chained uronides was more rapid than that of the oligouronides. Trigalacturonide inhibited the breakdown of the larger polyuronides, but digalacturonic acid was without effect. It is interesting to note that the pH optimum for the polyuronide hydrolysis was 5.0 but that it fell to 3.4 for the oligouronides. The authors draw attention to the danger of postulating two enzymes from these observations and question the existence of three different enzymes in the *A. foetidus* preparation as claimed by Dingle *et al.* (98). Demain & Phaff have now revised the earlier statement of Phaff & Luh (99) that the enzyme could not attack the trigalacturonide. The earlier observation was made at pH 5.0 before it was realized that the pH effected the hydrolysis of short- and long-chain uronides differently. It should be recognized, however, that it is by no means established that yeast polygalacturonidase is a single enzyme or that Dingle *et al.* are not dealing with several. Demain & Phaff have postulated on the basis of

specificity studies with the yeast enzyme on pectinic acids of various methoxyl contents [made by Luh & Phaff (100)] that for enzymatic hydrolysis to proceed, at least two neighboring free carboxyl groups must exist, and these two neighboring units, with the susceptible bond between them, must be remote from one of the free end groups. Which end this is awaits determination.

McCready & McComb (101) using fungal polygalacturonase have also effected a separation of the hydrolysis products, including mono, di-, and triuronides. They found the rate of hydrolysis of the di- and triuronides to be about 5 per cent of that of polyuronides ranging from 10 to 200 units in length. Since digalacturonic acid is split by their enzyme, the mechanism proposed for the yeast enzyme by Demain & Phaff is not applicable here McCready & Seegmiller (102) found that purified citrus pectinesterase does not attack the mono- or dimethyl esters of digalacturonic acid, the diester of methyldigalacturonic acid, nor the triester of methyltrigalacturonic acid. However polygalacturonic acid polymethyl esters with as few as 10 uronic acid residues are readily hydrolyzed. Purified fungal polygalacturonase tested against a variety of simple substrates hydrolyzed all those which had two adjacent uronic acid residues whose carboxyls were unesterified.

The difficulty of interpretation when one deals with a mixture of pectic enzymes and an ill-defined substrate can be further increased if insufficient regard is taken of the interdependence of pH optimum, electrolyte concentration in general, and Na^+ concentration in particular. The influence of these factors has been described by Schubert (103).

Alginase.—A partial separation of the end products of the action of alginase on algin has been made by Kooiman (104) who obtained some free mannuronic acid and a number of oligomannuronides through paper chromatography. The enzyme which was produced by an unnamed bacterium grown on algin, did not attack either high- or low-methoxyl pectin. Pectic acid was not tried.

A notable example of the use of an ion exchange resin for the purification of an enzyme is that described by Högberg (105). Crude hyaluronidase from bull testis was purified 100-fold by a single absorption and elution on an Amberlite XE-64 column. Bacterial hyaluronidase was not absorbed. Rasmussen (106) has also employed an ion exchanger, IRC-50, to achieve a 20-fold purification of testicular hyaluronidase in one pass, with a 64 per cent recovery.

Various preparations of partially purified bovine testis hyaluronidase do not show the same isoelectric point and sedimentation constant. The behavior of the enzyme on purification has led Malmgren (107) to ascribe these anomalies to the behavior of the carrier material. He has concluded that the correct molecular weight of the enzyme is about 11,000.

Mathews (108) has correlated the inhibition of testicular hyaluronidase by a variety of sulfated alcohols with the concentration of inhibitor necessary to achieve the formation of micelles. This worker suggests that these micelles,

which are positively charged aggregates, resemble chondroitin-sulfuric acid and thus cause inhibition. The inhibition, however, could not be reversed by dilution.

Tissue hyaluronidase differs from bacterial and testicular hyaluronidase as Gibian (108a) has found, in that it shows a different ratio of release of acetyl glucosamine to decrease in viscosity. The pH optimum of the tissue enzyme is also unusually low, being near 3.

Weissmann *et al.* (109) have succeeded in separating seven oligosaccharides from a digestion mixture of umbilical cord hyaluronic acid by testicular hyaluronidase. These range from di- to tetradecasaccharides and are made up of the same repeating unit found in hylauronic acid, namely glucuronidoacetylglucosamine.

Glucuronidase.—Highly purified β-glucuronidase of calf spleen or liver origin suffers an inactivation on dilution which can be arrested and even reversed by the presence of egg albumin [Bernfeld *et al.* (110)]. Of a number of substances tested as protective agents desoxynucleic acid and chitosan were most effective. β-glucuronidase, active on quinolyl-α-glucuronide, is reported by Bellett (111) to be in the common snail. Robinson *et al.* (112) find glucuronidase in the crop of the locust.

Diphosphopyridine nucleotide transglycosidase.—The cleavage and the exchange of the nicotinamide moiety of DPN[3] is catalyzed by DPN-transglycosidase (DPN-nucleotidase). The earlier work of Zatman and co-workers has shown that these enzymes may be divided into two classes depending on whether or not they are inhibited by INH.[2] These workers have further shown that the INH-insensitive enzymes catalyze the exchange of the nicotinamide moiety with INH to form the INH analogue of DPN, but they were not at first able to show that the INH-sensitive enzyme could do this. They also found that the INH analogue of DPN inhibited only the INH-sensitive enzymes. The recent demonstration by Goldman (113) that a sensitive enzyme (beef spleen) could also produce this analogue affords an explanation of the inhibiting action of INH. The sensitive enzyme apparently makes enough of the INH analogue to inhibit itself. The enzyme is, as it were, hoist by its own petard. Zatman *et al.* (114, 115) have confirmed Goldman's finding and have presented further detailed information regarding the behavior of these enzymes.

Sulfatase

Roy has described (116, 117, 118) the differentiation of two aryl sulfatases present in mammalian livers. He found both activities to be associated with the mitochondrial fraction. On the other hand, Dodgson *et al.* (119), also working with aryl sulfatase, found most of the activity in the microsomal fraction. This discrepancy was finally explained by Roy (120) and by Dodgson *et al.* (121) on the basis that they were dealing with sulfatases of different specificities. Thus both groups were able to repeat Roy's work when they used his substrate, nitrocatechol sulfate. Similarly the original conclusions of

Dodgson *et al.* were borne out when β-acetylphenyl sulfate was employed.

Dodgson & Spencer (122) have found a heat-stable, nondialyzable factor in human serum which promotes the hydrolysis of *p*-nitrophenyl sulfate. They (123) also report the visceral hump of the periwinkle to be a source of arylsulfatase, glucosulfatase, and chondrosulfatase. The "aryl" nature of the specificity of the arylsulfatase in "Clarase" is illustrated by Dzialoszynski's (124) finding that it splits α- and β-naphthylsulfate and 3,5-dimethylphenylsulfate but does not attack cyclohexylsulfate.

Methyl Transfer

S-Adenosyl methionine.—A more general function for S-adenosylmethionine is indicated in addition to its function as the immediate methyl donor for nicotinic acid. It has now been established that it plays the same role with respect to guanidoacetic acid. The enzyme which catalyzes this later reaction, guanidoacetate methylpherase, has been isolated and partially purified from pork liver by Cantoni & Vignos (125). This enzyme appears to be a sulfhydryl protein. There is no evidence that folic acid is involved in this transfer although the presence of this compound or a derivative has not been excluded. In view of the recent findings concerning the mechanism of tetrahydrofolic acid in one-carbon transfers there is perhaps little reason to expect its participation. In addition to the demonstration that creatine was formed (125), S-adenosylhomocysteine has also been isolated and identified [Cantoni & Scarano (126)].

Methylation in fungi.—Challenger *et al.* (127) have studied the effectiveness of a number of potential methyl donors or precursors in forming dimethylselenide and trimethylarsine in cultures of *Aspergillus niger* and *Scopulariopsis brevicaulis*. Choline chloride, betaine, and formate were only slightly effective ranging from 1 to 5 per cent conversion to dimethylselenide or trimethylarsine; methionine was unexcelled, giving 25 to 95 per cent. A most interesting observation was that both optical isomers of methionine were active.

Methylation in higher plants.—Apart from the fact that various organisms may differ with respect to the final recipients of the methyl group, the evidence grows that much of the mechanism adduced by experiments with mammals and bacteria is generally applicable to all organisms. Dewey *et al.* (128) have now firmly established that the methyl group of nicotine in tobacco can come intact directly from methionine and that formate is not in the pathway. Ahmad & Karim (129) have shown that the methyl groups of choline can arise from methionine in the chick pea.

Methylation in yeast.—The formation of methylthioadenosine in *Torulopsis utilis* is dependent on methionine or methionine precursors, according to Schlenk & Tillotson (130). Here, too, D- and L-methionine were both effective. The fact that methylmercaptan could replace methionine was taken by these workers as an indication that the mercaptan was also a precursor of methionine. Methionine itself is the precursor for S-adeno-

sylmethionine, which is turn gives rise to methylthioadenosine. Parallel behavior is exhibited with ethyl mercaptan and ethionine to give ethylthioadenosine.

Enzymes Acting on Carboxylic Acids

Tricarboxylic acid-splitting enzymes.—The report of Campbell, Smith & Eagles (131) that crude extracts of *Pseudomonas aeruginosa* could convert *cis*-aconitate to glyoxalate and succinate is highly intriguing and deserves reiteration in view of recent developments. The preparation, which is made from sonically treated cells, can also catalyze the same 2–4 cleavage with citric acid. This would suggest that aconitase consists of two enzymes and that only the one which interconverts citric and *cis*-aconitic acids is present. Thus when the extract acted on one of these carboxylic acids the other could be found. However the same effect might be expected if a reversible cleavage reaction could occur with either acid. The results with purified pig heart aconitase obtained by Morrison (132) leave little doubt that in this case aconitase is only one enzyme. On the other hand Dickman & Speyer (133) have made the amazing observation that soluble aconitase obtained by freezing and thawing rat liver is more active than mitochondrial aconitase in catalyzing the conversion of citric and isocitric acid to aconitic acid but less active in converting isocitric acid to citric acid. Furthermore soluble aconitase has a pH optimum of 7.3 while mitochondrial aconitase has optima at pH 5.8 and 7.3. The further purification of *P. aeruginosa* extracts by Smith & Gunsalus (134) has indicated an even greater complexity of the problem, for they have obtained a fraction which cleaves only *iso*-citric acid and has no effect on citric or aconitic acid. Their enzyme, "isocitrase," shows an absolute requirement for a divalent metal which is best satisfied by Mg^{++} although Fe^{++}, Co^{++}, and Mn^{++} are also effective. Reduced glutathione or cysteine must also be present. It is odd that Campbell and co-workers working with an obviously cruder preparation found no "isocitrase" activity, since both groups of workers used the same strain. It may be that the system of Campbell *et al.* was deficient in the cofactors necessary for the "isocitrase." Saz (135) found that all three of the tricarboxylic acids were converted to succinic and glyoxylic acids by sonic extracts of *P. aeruginosa* and *P. fluorescens*, but he deduced that isocitric acid was here, too, the immediate precursor. Olson (136) reported that cell-free extracts of *Penicillium chrysogenum* cleaved citric acid to the 2- and 4-carbon acids. From the effect of specific inhibitors he inferred that the cleavage enzyme was specific for isocitric acid and accordingly named it D-isocitric lyase. Baker's yeast, *A. niger*, and various *Rhizopus* species also contain this enzyme. Smith & Gunsalus (private communication) have found the isocitric acid-cleaving enzyme to be widely distributed among the bacteria, being particularly high in *Pseudomonas* and *Azotobacter* genera. The citrodesmolase of *Escherichia coli* has been studied by Wheat *et al.* (137).

5-Dehydroquinase.—This enzyme, which reversibly dehydrates 5-de-

hydroquinic acid to dehydroshikimic acid, has been isolated from *E. coli*, strain W, and partially purified and characterized by Mitsuhashi & Davis (138). It is completely specific for the two named substrates, but without effect on malate, isocitrate, citrate, or quinic acid. It is inhibited by Cu^{++}, Zn^{++}, and Fe^{++} and appears to require no metal activator. It has a broad pH optimum at 8.0. The absorption at 234 mμ by dehydroshikimic acid provides the basis for a simple assay procedure. At equilibrium there is about 15 times as much dehydroshikimic acid present as dehydroquinic acid. The

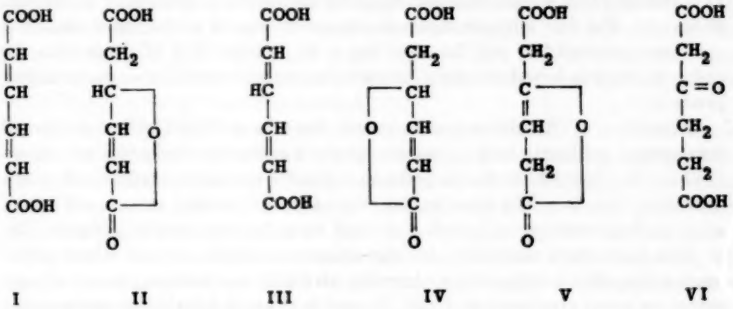


FIG. 1. Intermediates in conversion of *cis-cis*-muconic acid to β-ketoadipic acid.

enzyme is also present in *Aerobacter aerogenes*, *Saccharomyces cerevesiae*, *Euglena gracilis*, peas, and spinach leaves but not in guinea pig liver.

Transformation of cis-cis-muconic acid to β-ketoadipic acid.—The microbiologists are not only making a successful onslaught on the problem of the metabolic formation of benzenoid compounds, but they have also struck an impressive blow on the obverse of the problem, the metabolic degradation of such compounds. Several of the links in the chain of reactions whereby pyrocatechol is converted to β-ketoadipic acid have been further delineated by Sistrom & Stanier (139) who have isolated two pertinent enzymes and described their reactions. These enzymes have been obtained from *Pseudomonas fluorescens* (strain A. 3.12) and were induced by mandelate. Aside from their interest in connection with the metabolic scheme in question, they are fascinating by virtue of the unusual type of action they catalyze (see Fig. 1). *Cis-cis*-muconic acid (I) (known to arise from pyrocatechuic acid by enzymatic oxidation) is converted by the "lactonizing" enzyme to (+)-α-carboxymethyl-Δ^{α}-butenolide (II) in the presence of Mg^{++}. Ethylenediaminetetraacetic acid inhibits this enzyme. The difference in the absorption spectra of the two compounds affords a simple method of assay. The reaction is reversible, the equilibrium position being greatly influenced by pH, as might be expected. At pH 6.0 virtually all of the product is lactone but at pH 9.0 about 97 per cent is present as muconic acid. The pH optimum

for the forward reaction lies between 7 and 8; at pH 8.0 the K_M for muconic acid $= 9.8 \times 10^{-5}$ *M*. If the lactonization is preceded by an initial dehydration, the subsequent lactonization must be very rapid, for no such intermediate could be detected. *Cis-trans*-muconic acid (III) is very slowly attacked. However the (−)-lactone (IV) does attain equilibrium, although slowly, with *cis-trans*-muconic acid. The *trans-trans* isomer is entirely inactive. Conversion of the (+)-lactone (II) to β-keto adipic acid (VI) is catalyzed by the "delactonizing" enzyme, which does not act on the (−) lactone (IV). Presumably II rearranges to V before the opening of the lactone ring, according to Sistrom & Stanier. The last enzyme does not appear to have a metal requirement, it operates optimally at pH 7.5, and has a $K_M = 7.8 \times 10^{-5}$ *M*. Here also the assay method is based on the different absorption spectra of substrate and product.

Fumarase.—The kinetics of fumarase have been the object of concerted treatment by Alberty and his co-workers in a series of closely related papers (140 to 143). They have developed and applied a theoretical treatment which considers ionic bonding sites on the enzyme, catalytically active and otherwise, and interaction with buffer as well as substrates and inhibitors. The authors have thus accounted for the anomalous variations of initial maximum velocities and K_M values observed with different buffer concentrations, substrate concentrations, and pH. A modification of existing procedures for the crystallization of fumarase is described. The physico-chemical properties of the enzyme agree with those of fumarase obtained in the older way. The M is 220,000 and the turnover number for malate to fumarate is 100,000 moles per min. at 25°C. (144).

The action of the following inhibitors on pig liver fumarase in the conversion of fumarate to malate has been studied by Jacobsohn (145): citraconate, itaconate, crotonate, aconitate, and succinate. Acetate and methyl fumarate were without effect as inhibitors.

The question as to the distribution of enzymes within the cell is becoming increasingly important in view of the concept of spatially organized metabolic systems. The enzymes fumarase and aconitase, integral components of the Krebs cycle, are expected to be associated with the mitochondria, and yet they are often found to a considerable extent in the soluble portion of a tissue extract. This is an artifact in preparation and is supported by Nossal's (146) demonstration that the distribution between the soluble and granular fractions of disintegrated yeast cells is a function of the disintegration time.

Glycine acylase.—The enzyme which Schacter & Taggart (147) previously showed to catalyze the formation of hippurylamide from hippuryl CoA and glycine has now been extensively purified from the acetone powder of beef mitochondria and has been found to be very specific for glycine but less fastidious with respect to the CoA compound. It uses a number of aliphatic acyl CoA compounds from C_2 to C_{10}, as well as aromatic acyl derivatives.

Malonic acid decarboxylation.—Hayaishi (148) who found that malonic

acid decarboxylation by *P. fluorescens* (strain TR-23) requires ATP and CoA, and that both acethydroxamic acid and malonomonohydroxamic acid are formed when hydroxylamine is present has now further clarified the operation of the malonic decarboxylase system (149). According to his mechanism, malonate is first activated by ATP and CoA. The malonyl CoA then undergoes decarboxylation but the acyl~S-CoA bond is conserved, for acetyl CoA is formed. The acetyl CoA can then activate another molecule of malonate. Thus only catalytic amounts of ATP were needed with a partially purified preparation of the carboxylase. Wolfe *et al.* (150, 151, 152), have independently obtained somewhat similar results and have shown further that Mg^{++} is required for the decarboxylation. It is of interest to note that they were able to show that a di-activated malonate (CoAS~ $COCH_2CO$~SCoA) was also formed.

Ellfolk (153) has tested a long list of potential substrates or inhibitors of aspartase from *Propionibacterium petersonii*. Of the several amino compounds tried only L-aspartic acid was deaminated. Among the more potent inhibitors were: oxalate, citrate, 1,2-ethanedisulfonic acid, and α-hydroxy-β-sulfopropionic acid. L-Cysteic acid and D-asparate were without effect. Ellfolk has inferred the enzyme-substrate combination is ionic in nature. He postulates a two- or three-point attachment involving an alkaline earth metal and the formation of a ring.

Enzymes Acting on Ureidyl Substrates

Phosphorylative decarboxylation of citrulline.—Korzenovsky & Werkman (154, 155, 156) have reported that the L-citrulline phosphorylase present in cell-free extracts of *Streptococcus lactis* decarboxylates citrulline in accord with the following equation:

$$\text{L-citrulline} + \text{ADP} + PO_4^{\equiv} \rightleftarrows \text{L-ornithine} + \text{ATP} + CO_2 + NH_3$$

They have shown the reaction to be reversible by using $C^{14}O_2$ and have demonstrated a net synthesis of citrulline in the presence of ATP. Neither carbamyl-L-glutamic acid nor acetyl-L-glutamic acid was required. On the other hand, Slade *et al.* (157) have investigated what apparently is a similar system from *Pseudomonas sp.* which differs in some important matters and betokens either the formation of intermediates or the participation of side reactions. Although their preparation had little adenosinetriphosphatase and no urease or "ornithinase" activity the ratio of ATP formation to citrulline destruction was only 0.7 in high phosphate concentration and fell to 0.3 to 0.4 in minimal phosphate. In the latter case more phosphate was esterified than could be accounted for as ATP. The *Pseudomonas* extract possessed no myokinase so it was feasible to show that AMP[2] could replace ADP as a phosphate acceptor. These workers refer to their system as citrulline ureidase.

Knivett (158) working with *S. faecalis* reached conclusions substantially in agreement with Korzenovsky & Werkman although he did not find ex-

actly the stoichiometric relations required. His preparation was able to use AMP, but since myokinase was present he assumed the primary acceptor to be ADP. He has calculated that under physiological conditions this reaction could release enough energy to make one pyrophosphate bond. In agreement with the other workers he has shown that arsenate can replace phosphate and adenylate (159).

The participation of "compound X" of Grisolia & Cohen (160) in catalytic amounts is a possibility that cannot be discarded. Perhaps the lack of effect of carbamyl glutamic acid (a precursor of "compound X") observed by Korzenovsky & Werkman signified saturation of the system by the "coenzyme." The existence of a "phosphatase" which destroys "compound X" has been shown in rabbit tissues by Grisolia & Marshall (161). This may perhaps prove a useful tool for investigating the bacterial system.

The mechanism that is finally promulgated to explain the reaction will have to account for Stulberg & Boyer's (162) observation that CO_2^{18} is formed when O^{18} -labeled phosphate is used. This strongly suggests that at some point an anhydride between phosphate and free or substituted carbonic acid must have been formed with the phosphate ultimately going to the acceptor adenylate. The possibility cannot be excluded that some other oxygen-containing intermediate was formed from the phosphate which then passed the oxygen on to the carbon of (probably substituted) carbonic acid.

Formation of carbamyl aspartic acid.—Compound X has been given a role on still another front, the synthesis of a carbamylaspartic acid (or ureidosuccinic acid). The latter compound is well-established as a precursor of orotic acid. Acetyl-compound X, labeled by making it enzymatically from acetyl-glutamic acid and $C^{14}O_2$, was shown by Lowenstein & Cohen (163) to form labeled carbamylaspartic acid in the presence of aspartic acid and rat liver enzyme. The conversion of citrulline to orotic acid in the rat liver, reported by Smith & Stetten (164), is believed by Lowenstein & Cohen to occur via compound X and carbamylaspartic acid. The discovery by Reichard (165) that rat mitochondria makes carbamylaspartic acid from aspartic acid, NH_3, and CO_2 if carbamylglutamate or glutamate is present, was also cited in support of this mechanism.

"Guanidine-decomposing" enzyme.—The breakdown of various guanidine derivatives by some strains of *Mycobacterium* occurs through the combined action of a guanidine-decomposing enzyme which produces urea and an amine, and urease [Zeller *et al.* (166)]. In the presence of urease the two enzymes can be distinguished by the differential inhibition of urease with Hg^{++} and the greater heat lability of the guanidine-decomposing enzyme. The latter can hydrolyze agmatine, arcaine, and 1-guanidino-2-(p-hydroxyphenyl)-ethane. It is distinct from arginase and other enzymes which attack guanidine derivatives [Zeller & Kirchheimer (167)].

Urease.—This enzyme has been purified from *Bacillus pasteuri* by Larson & Kallio (168), and although not crystallized it had an activity of 165,000

Sumner units per gm. compared to 133,000 for crystalline jack bean urease. The substrate specificity of the two enzymes is the same but the bacterial enzyme has a lower energy of activation. However, as has been noted before with soybean and jack bean urease the Arrhenius plot shows a break. This has been interpreted as representing a reaction with an energy of activation of 9900 calories above 33°C. and 4400 calories below this temperature. The organism is remarkably rich in urease, containing 1 per cent on a dry weight basis.

Shaw has pointed out that the observed order of toxicity of a series of metal ions to urease is well correlated with the insolubility of their sulfides (169). According to Jacobsohn & deodata de Azevado (170) urease activity is slower in D_2O than in H_2O.

The intensive production of hydrions by the gastric mucosa cannot, after all, be connected with urease activity. Kornberg *et al.* (171) have shown, first that gastric urease (at least in the cat) is entirely of bacterial origin and secondly it is present in too low a concentration to exert any significant effect.

Arginase.—The controversy regarding the ability of Co^{++} to activate liver arginase has again come to life. Bach & Whitehouse (172) describe a purified preparation that does not respond to Co^{++}. On the other hand, a preparation obtained from *Dolichos lablab*, (obviously very crude) does respond well to Co^{++}, Fe^{++}, or Mn^{++}. Vaidyanathan & Giri (173) have found the enzyme in the seedling in free and bound form. Prolonged dialysis or acetone precipitation reduces the activity of these preparations. No evidence is presented, however, to support the claim that this is caused by the removal of an essential cofactor.

Amino Acid Decarboxylase

Histidine decarboxylase.—The apoenzyme of histidine decarboxylase from *Lactobacillus* 30a grown on a medium deficient in vitamin B_6 has been found to require pyridoxal phosphate and Fe^{+++} or Al^{+++} by Guirard & Snell (174). The significance of an unequivocal demonstration of a trivalent metal requirement, in connection with the metal-substrate-pyridoxal chelate mechanism proposed by Metzler, Ikawa & Snell (175) is obvious. Incidentally it should be noted that these authors have taken cognizance of the discovery by Mandeles *et al.* (176) that in the enzymatic decarboxylation of lysine, tyrosine, or glutamate in D_2O only one D is found on the amino-bearing carbon atom. They therefore postulate that the imino bond formation occurs simultaneously with the release of the carboxyl group.

5-Hydroxytryptophan decarboxylase.—5-Hydroxytryptamine which has been regarded as having its origin in 5-hydroxytryptophan is potentially an important physiological regulator. Hence it is of interest that Clark *et al.* (177) have obtained an enzyme from a variety of animal tissues which can decarboxylate 5-hydroxytryptophan to give this product. The enzyme, whose concentration from acetone-dried hog and guinea pig kidney powder is described, is highly specific, attacking only 5-hydroxy L-tryptophan of a

number of related compounds. While it is presumed to be a pyridoxal phosphate enzyme it was not possible to show such a requirement nor was it possible to reverse the inhibition caused by carbonyl reagents with pyridoxal phosphate. The enzyme has an unusually high affinity for its substrate for the rate is still at a maximum at 5×10^{-5} M, the lowest concentration which permitted measurement.

Najjar & Fisher (178) have purified glutamic acid decarboxylase from *E. coli*. Their preparation will be of interest to those who wish a specific glutamic acid reagent because it is free of glutaminase, aspartic acid decarboxylase, and glutamic acid racemase.

Enzymes Attacking Sulfur Compounds

Rhodanese.—This enzyme which has been crystallized by Sörbo (179) from beef liver has a molecular weight of 37,000 and a turnover number of 20,000 (180). The reaction which it catalyzes has an energy of activation of 7900 calories per mole and is irreversible. Sulfhydryl and disulfide groups are present in the enzyme. It shows a broad pH optimum of 3 to 9 (181). In addition to catalyzing the formation of thiocyanate from cyanide and thiosulfate, the enzyme can also use aryl and alkyl thiosulfonates. Sörbo has proposed a reaction mechanism in which the thiosulfate combines with the enzyme through an opened disulfide bond. The enzyme-substrate complex then reacts with cyanide to form sulfite and thiocyanate, and the enzyme is returned to its original state.

Sörbo (182), using the purified enzyme, demonstrated that rhodanese could not utilize β-mercaptopyruvic acid in place of thiosulfate as had been claimed by Wood & Fiedler (183) who used crude liver homogenate. Sörbo suggested that the observation was a result of the action of the desulfurizing enzyme of Meister *et al.* (184) on the mercapto acid to give free sulfur; this then reacted with the sulfhydryl and disulfide groups in the tissue proteins to produce thiocyanate.

Cysteine desulfurase.—The observation was made some years ago by Desnuelle *et al.* (185) that *Propionibacterium pentoaceticum* liberated H_2S from L- or D-cysteine at equal rates. Saz & Brownell (186) have found that preparations from *E. coli* (strain E-26 and also from an aureomycin-resistant strain) attack cysteine to produce H_2S, pyruvic acid, and NH_3 but that D-cysteine is greatly preferred. The chlortetracycline (aureomycin) resistant strain was more than twice as active as E-26. Neither D- nor L-amino acid oxidase was present.

Biocytinase.—Wright *et al.* (187) have found a hydrolase in human blood which cleaves biocytin (ϵ-N-biotinyl-L-lysine) to give biotin and lysine. The enzyme, which is apparently distinct from ordinary proteinases, also hydrolyzes biotinamide and may be responsible for the appearance of biotin in urine following administration of biocytin.

Methionine demercaptodeaminase.—The enzyme which is obtained from

Clostridium sporogenes, produces methyl mercaptan, pyruvic acid, and NH_3 from L-methionine and requires pyridoxalphosphate [Wiesendanger & Nisman (188)].

Penicillinase.—Pollock & Torriani (189) have obtained a highly purified penicillinase from *B. cereus* NRRL 569 which has a turnover number of 1.3×10^5. Its M is estimated to be 56,000. Baudet & Hagemann (190) have also obtained a highly purified preparation which they crystallized at a cost in specific activity. A spectrophotometric assay for this enzyme based on the disappearance of absorption at 240 mμ when penicillin is converted to penicillinoic acid has been devised by Baudet & Cherbuliez (191).

Transaminases

A suvey of the literature shows an impressive growth in the catalogue of tissues containing various transaminases. While the presence of these enzymes is of considerable importance in the formation and interconversion of amino acids in these tissues it is not practical to list them here. The principle of comparative biochemistry is fortunately a great simplifying factor in this domain. A few instances of work of special interest will be cited here.

Reactions with arginine.—The study of the behavior of arginine in a crude rat liver glutamine transaminase-deamidase preparation is beset with the difficulty that a high arginase activity is present. Meister (192) has met this problem by determining first of all the behavior of the arginase reaction product of arginine, namely ornithine, and then studying the reverse reaction by starting with the α-keto analogue of arginine. Ornithine transaminates with a number of keto acids, but as found by Fincham (193) for *Neurospora* it tranfers its ω- rather than its α-amino group and forms glutamic-γ-semialdehyde. The products obtained when α-keto-δ-guanidovaleric acid reacted with glutamine at pH 8.2 (urea, NH_3, glutamate, and glutamic-γ-semialdehyde) are consistent as Meister points out, with the following: the initial formation of arginine, its breakdown by arginase to ornithine, and its transamination with α-keto glutarate formed from the glutamine. At pH 6.0 the α-ketoglutarate-ornithine reaction is not operative and so ornithine is formed, but not glutamate or glutamic-γ-semialdehyde. That arginine is very likely the initial product is demonstrated by the fact that the keto analogue of nitroarginine transaminates with glutamine to yield nitroarginine, which is resistant to arginase hydrolysis.

Reactions involving glutamine.—The glutamine-α-keto acid transaminase system in a purified preparation from rat liver forms α-keto glutaric acid and ammonia rather than the expected amino acid amide (α-ketoglutaramic acid). It has been tested for its specificity with respect to the amino acid amide donor by Meister (194). Of the several acid amides tested only γ-methylglutamine and γ-methyleneglutamine were active in addition to glutamine. The two compounds did not yield ammonia. With γ-methylglutamine, α-methyl-α-ketoglutaramic acid was isolated. These findings

serve to verify the original belief that the system contains two enzymes, one catalyzing the conventional transamination and the other deamidating α-ketoglutaramic acid.

The reversibility of the glutamine-α-keto acid transamination was not amenable to test because of the highly active amidase present, and because the α-ketoglutaramic acid tends to exist largely in its nonketo modification under the test conditions. On the other hand, the analogous system, asparagine-α-keto acid transaminase, also present in rat liver, was shown to be reversible with α-keotsuccinamic acid and a variety of α-amino acids [Meister & Fraser (195)]. In this instance, too, a highly active α-keto -γ-amidase was present, but as a result of the fortunate circumstance that it was saturated by substrate at a lower concentration than the transaminase, it was possible with excess substrate to increase transamination more than deamination. Thus the yields of asparagine when the substrate was supplied at 1.00, 0.66, and 0.33 of the optimum concentration were relatively 1.0, 0.5, and a trace respectively.

Coenzymes of transaminase.—Meister, Sober & Peterson (196) have repeated in greater detail the observation that pyridoxaminephosphate is equivalent to pyridoxalphosphate in activating pig heart glutamic-aspartic transaminase. Activation of the apoenzyme by either of these substances requires time; pyridoxaminephosphate requires twice as long as pyridoxalphosphate. Once the apoenzyme has been activated it does not lose its activity even on prolonged dialysis and then retains about 100 times as much coenzyme as is required by the enzymatically active sites. The excess coenzyme is apparently bound to nonspecific sites. Reconstituted enzyme is not effected by pyridoxinephosphate or desoxypyridoxinephosphate. The apoenzyme, if preincubated with these substances, cannot then be fully activated when presented with its normal coenzymes.

Enzymes Acting on γ-Glutamyl Peptide Bonds

Glutamyl synthetase.—The kinetics of the forward and reverse reaction in which glutamine is formed from glutamic acid and NH_3 in the presence of ATP as shown below has been studied by Levintow & Meister (197) with a purified pea enzyme.

$$\text{glutamic acid} + NH_3 + \text{ATP} \rightleftarrows \text{glutamine} + \text{ADP} + PO_4^{\equiv}$$

The free energy of the reaction has been determined from the equilibrium constant. Using this value together with a reasonable assumption for the $-\Delta F^\circ$ of hydrolysis of glutamine, a $-\Delta F^\circ$ of -8000 calories, which is somewhat lower than the generally accepted value, is obtained for the hydrolysis of the first phosphate of ATP. A remarkable property of the system is that it can use D-glutamic acid as well as the natural isomer, but whereas inorganic P^{32} phosphate is incorporated into ATP when L-glutamate is the substrate, such is not the case with D-glutamate. Webster & Varner (198)

have interpreted the incorporation of P^{32} into ATP, which they observed in the same system, as proof of the following mechanism:

$$\text{Enzyme} + \text{ATP} \rightleftarrows \text{Enzyme-P} + \text{ADP}$$
$$\text{Enzyme-P} + \text{Glutamic acid} + NH_3 \rightleftarrows \text{Enzyme} + \text{Glutamine} + P$$

They found an increased incorporation into ATP when NH_3 was omitted, which they explain by postulating that the concentration of Enzyme-glutamic acid remains greater when there is no NH_3 present. However, the fractions of available P^{32} which were incorporated into ATP were, respectively, 0.12 per cent (glutamate+NH_3 omitted), 1.1 per cent (complete system), and 0.29 per cent (NH_3 omitted). An unfavorable equilibrium constant for the formation of this intermediate could account for the low values obtained in short time experiments. If Levintow & Meister's conclusion is accepted that only one enzyme is involved, any theory to explain the behavior of the enzyme has to account for the incorporation of P^{32} into ATP during the forward reaction with L-glutamic acid, but not with D-glutamic acid. It must also explain why ADP is required for the asenolysis of L-glutamine.

γ-Glutamylcysteine synthetase.—Wheat germ extracts which catalyze the synthesis of γ-glutamylcysteine from glutamic acid and cysteine in the presence of ATP, Mg^{++}, and K^+ also promote an exchange of inorganic phosphorus with ATP phosphorus, if glutamic acid is present [(Webster & Varner (199)]. The presence of cysteine slows up the exchange. A small but measurable exchange of P^{32} between ATP and ADP^{32} occurs in the absence of the amino acids. These authors have concluded that the reaction mechanism involves an enzyme~P complex as the first step and just as in their glutamyl synthetase experiment glutamylphosphate cannot be an intermediate. They also make the point that their preparation of glutamyl synthetase from peas can also catalyze the synthesis of γ-glutamylcysteine and are thus led to the interesting suggestion that the two activities might simply be the property of a single enzyme, having a broad specificity with regard to glutamyl acceptors.

Glutaminase I.—This enzyme which is associated with the mitochondria of the cells in which it occurs has been brought into solution from hog kidney tissue by a butanol treatment [Otey *et al.* (200)] and then appears to be less stable than in the bound form. It may be stabilized by borate. Both insoluble and soluble preparations require arsenate, sulfate, or phosphate for activation or stabilization. Since the activities of the soluble preparation against L-glutamine, L-asparagine, and L-isoglutamine respectively are not equally stable, a multiplicity of enzymes rather than a broad specificity seems to be indicated.

Activity in Unnatural Optical Isomers

Asparaginase.—With the increasing recognition of the existence of "unnatural" amino acids in bacterial products it should no longer occasion wonder that "unnatural" enzymes are found and yet the presence of such

enzymes continues to be an intriguing matter, although the D-amino acid oxidases are commonplace. Altenbern & Housewright (201) have described both D- and L-asparaginases in *Brucella abortus* (strain 19, smooth). Both enzymes are found in the sonic extracts of this organism and have been separated by fractional precipitation with acetic acid. They are both sulfhydryl enzymes, but otherwise are distinguishable by their different pH optima. The D-asparaginase is active over a wide pH range while the L-enzyme has a sharp pH optimum at 8.0. Moreover the enzymes are unequally resistant to freezing and thawing and have different K_M values. L-asparagine is not particularly inhibitory to D-asparaginase. There is no basis for invoking a racemase to explain the dual activity of this preparation.

Alanine racemase.—The fact that the germination of some bacterial spores is stimulated by L-alanine and inhibited by D-alanine has led to the hypothesis that alanine racemase, present in some spores, might be concerned with the regulation of germination. However, Church, Halvorson & Halvorson (202) have discovered that spores of *Bacillus megatherium* contain the racemase but do not require L-alanine for germination, whereas the spores of *Bacillus globigii* lack this enzyme but do require alanine. Furthermore if *B. megatherium* spores are brought to pH 11.3, at which pH the racemase is completely inactive, they do not lose their ability to germinate. The authors conclude, therefore, that the above hypothesis is untenable.

The heat resistance of bacterial spores is reflected in a corresponding heat-resistance of some of the enzymes they contain. This resistance disappears when the spores become vegetative. Stewart & Halvorson (203) found that the alanine racemase of *Bacillus terminus* spores underwent only 10 per cent inactivation at 80°C. for 2 hr. whereas the same enzyme from vegetative cells was completely destroyed in 15 min. The heat resistance of the spore enzyme is associated with its particulate nature for the resistance to heat was lost by solubilization. Lawrence & Halvorson (204) found that a similar situation obtains for the catalase present.

Lactic acid dehydrogenase.—Further evidence that the effect of lactic acid racemase is not the net result of simultaneous L- and D-lactic acid dehydrogenase activity has been furnished by Kitahara *et al.* (205), who obtained a racemase preparation from *Lactobacillus plantarum* which was entirely free of lactic acid dehydrogenase.

D- *and* L-*transaminases.*—A fascinating investigation of classical proportions by the group at Camp Detrick has solved the problem of how peptides containing unnatural optically active amino acids arise, in the case of the γ-glutamyl peptide elaborated by *Bacillus subtilis*. An enzyme produced by the organism catalyzes with great efficiency a transpeptidization between two molecules of γ-D-glutamyl-D-glutamic acid to yield a tripeptide and a free amino acid (and under some conditions, larger peptides). The amino acid bearing the free γ-carboxyl can also be exchanged by the same enzyme for glutamic acid in solution. D- or L-peptides serve equally well [Williams &

Thorne (206)]. The unnatural configuration is introduced by the action of alanine racemase, which together with the available alanine might be considered a reservoir of "right handedness." The transfer of this "right handedness" to other amino acids can then be accomplished by D-transaminase, which is present and can form D-glutamate from α-ketoglutarate and D-alanine or D-aspartate. Like its L-counterpart, this enzyme requires pyridoxal phosphate [Thorne, Gómez & Housewright (207)]. The breakdown of the naturally occuring peptide with its mixture of D- and L-glutamyl residues to give free glutamic acid and a mixture of oligopeptides can be accomplished with an enzyme prepared from the organism [Thorne *et al.* (208)].

Miscellaneous Enzymes

Iodinase.—Fawcett & Kirkwood (209) have identified as monoiodotyrosine the product in the previously demonstrated iodination of tyrosine by thyroid gland homogenates. They have now prepared a water-soluble enzyme which catalyzes this reaction (210). Its specificity has been tested with a range of compounds. The following reacted positively: (rate with L-tyrosine, =100) D-tyrosine, 60; 3-(*p*-hydroxyphenyl)-propionic acid, 71; N-acetyl-D, L-tyrosine, 65; 3-fluorotyrosine, 29; *p*-hydroxybenzoic acid, 23. Among the substances which did not react was *p*-hydroxyphenylacetic acid. The investigators postulate that the requirements of specificity can only be met by compounds in which the carboxyl group can exist in the same plane as the benzene ring when the carboxyl and phenolic groups are maximally separated. The enzyme also catalyzes the exchange of I^{131} with monoiodotyrosine, but it does not catalyze removal of iodine. It is possible that iodinase is an oxidative enzyme.

Xanthurenicase.—This enzyme, which catalyzes the destruction of xanthurenic acid, has been found by Kotake *et al.* (211) in aqueous extracts of rat and rabbit liver. The mode of action of the enzyme is unknown. Xanthurenicase has a pH optimum of 7.2 and is inhibited by KCN, NaF, and iodoacetate. The destruction of xanthurenic acid ceases when 30 to 50 per cent has reacted. Paper chromatography of the reaction products has revealed, in addition to the unreacted substrate, a second substance which is distinguished by an absorption spectrum have a maximum at 270 mμ and a minimum at 250 mμ.

Tryptophanase.—As prepared from *E. coli* the enzyme split indole from L-tryptophan, but was without action on a number of related indole derivatives [Gooder & Happold (212)]. The authors concluded from the efficacy of some of these compounds as inhibitors and on the basis of other considerations that an indole nucleus with an unsubstituted nitrogen was a necessary but insufficient condition for union of the substrate with the enzyme.

Nicotinamidase.—Oka (213) has found that only a portion of the activity associated with yeast powder is made soluble by homogenization. It is possible to obtain nicotinamidase free of asparaginase activity if autolyzed yeast

is used. Hg^{++} and Cu^{++} inhibit the enzyme, which has a pH optimum at 7 to 7.5. Whether this activity is attributable to a specific nicotinamidase cannot be stated since its specificity was not otherwise investigated. A 15-fold purification has been achieved with the aid of calcium phosphate gel (214).

Enzymes acting on ureidosuccinic acid.—Two new and interesting enzymes concerned with pyrimidine metabolism (which is not covered here) are dehydro-orotase and 5-carboxymethylhydantoinase which catalyze reactions A and B respectively (see Fig. 2). The enzymes have been obtained in

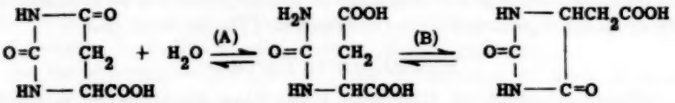


FIG. 2. The action of dehydro-orotase and 5-carboxymethylhydantoinase on L-ureidosuccinic acid.

soluble form from *Zymobacterium oroticum* by Lieberman & Kornberg (215). The equilibrium reaction constants for A and B were determined to be 1.9 in both cases.

Transoxaminase.—A novel enzymatic reaction, the transfer of the oxime group, =NOH, from pyruvic acid oxime to acetaldehyde or acetone has been reported by Yamafuji *et al.* (216). This enzyme, found in silk worm larvae, is heat labile and has a pH optimum of 5.6. Yamafuji & Eto (217) have more recently extended this work to include ketoglutaric acid and have made positive identification of the products.

Creatinine formation.—Caputto (218) has found that washed rabbit muscle can transform creatine phosphate to creatinine in the presence of glucose-1-PO_4. This ability is lost on heating. Since the preparation contains phosphohexoisomerase and phosphoglucomutase it is not possible to decide if glucose-1-phosphate participates directly in the reaction. Caputto also reports that some glucose-1,6-diphosphate can be formed from creatinine phosphate and glucose-1-phosphate. This suggests that the presence of phosphorylated sugars would promote an accumulation of ATP which in conjunction with ATP-creatine transphosphorylase would tend to keep the creatine phosphate from being hydrolyzed to creatine.

Conversion of inositol to aromatic compounds.—The metabolic connection between inositol and aromatics, long hypothesized, gains substantial support from the pathway of flavanoid synthesis which Birch, Donovan & Moewus (219) have proposed after studying the behavior of a series of x-ray mutants of *Chlamydomonas eugametos*. Mesoinositol appears to be the precursor of phloroglucinol.

DDT-dehydrochlorinase.—The discovery by Sternburg *et al.* (220) that flies resistant to DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] contain an extractable enzyme which promotes the dehydrochlorination of DDT and related insecticides is intriguing because of the novel type of reac-

tion catalyzed and also for its implications in the matter of evolution and selection. Sternburg *et al.* (221) tested a number of strains of house flies and found that only the resistant strains contain the enzyme. Using aqueous extracts of acetone powder preparations these workers have observed that in addition to DDT, 1,1-dichloro-2,2-bis(*p*-methoxyphenyl)ethane was readily attacked. Those compounds in which *p*-tolyl and *p*-iodophenyl replaced the *p*-chlorophenyl group of DDT were but slightly attacked. Toxicity correlated well with susceptibility to enzyme attack. The DDT analogues in which the phenyl groups were unsubstituted or in which one of the chlorine atoms was borne in the *o* position did not act as substrates. DDT formed only one product, the nontoxic 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene. Resistance in flies to another type of chlorinated hydrocarbon, dieldrin, a naphthalene derivative, was not accompanied by DDT-dehydrochlorinase. The enzyme acts on the surface of the solid substrate and the DDT must be presented in semicolloidal form for optimum activity. In routine assays the DDT is supplied as a thin layer on minute glass beads. A most remarkable property of this enzyme is its inability to withstand ethanol in concentrations as low as 0.02 per cent.

Since DDT-resistance appears to be genetically transmitted it is likely that this character must exist, albeit with low frequency, in a normal pre-DDT population. It is difficult to imagine the function of such an enzyme prior to the existence of DDT. The specificity of this enzyme has been studied, however, only with a limited number of compounds, all of which are analogues of DDT. It is conceivable that its action on these highly artificial substrates is merely a consequence of a more general property.

Formylase.—Jakoby (222) has found that formylase from *Neurospora crassa* is remarkably similar to the enzyme originally described in the mammalian liver, in regard to its specificity. It catalyzes the deformylation of N′-formyl-L-kynurenine and several aromatic formylamines and does not act on formyl amino acids. Jakoby suggests the name "kynurenine formamidase" for this enzyme.

General

Mechanism of hydrolytic reactions.—An interesting generalization concerning the determination of the O–R bond split by hydrolytic enzymes has been drawn by Koshland & Stein (223) from a consideration of observations on the hydrolysis of isotopically labeled reactants and group transfer reactions. The behavior of invertase, acid and alkaline phosphatases, trypsin, and chymotrypsin is consistent with the idea that if in substrate, R′–O–R″, R′ meets the specificity requirement of the enzyme then the cleavage will occur between R′ and O. It is intriguing to speculate on the behavior of mixed acid anhydrides in the reactions of Bergmann *et al.* (75). (See also Phosphatases, Diesterases, this chapter.)

Technique.—While it is not intended to review developments in histochemistry here, attention is called to some admirable techniques for measur-

ing enzyme activities in extremely small quantities of tissue. Lowry *et al.* (224) describe the determination of aldolase, adenosinetriphosphatase, fumarase, acid phosphatase, alkaline phosphatase, and choline esterase on 10 μg. of tissue. Of special interest is their adaptation of a fumarase method described by the late J. F. Speck in which the malic acid formed is reacted with β-naphthol and sulfuric acid to yield a fluorescent product.

Sober & Peterson (225) have successfully applied cellulose columns to the separation and purification of leucine and alanine amidase and aspartic-glutamic transaminase.

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PROTEOLYTIC ENZYMES[1,2]

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Reviews of various aspects of the chemistry and activity of chymotrypsin and pepsin (66), of chymotrypsin and trypsin (103), of prolidase and leucine aminopeptidase (125), and of the digestive proteases (31) have appeared during the past year.

Isolation

Papain.—The isolation of crystalline papain from dried papaya latex has been described by Kimmel & Smith (80). The procedure is similar to that of Balls & Lineweaver (6) for the isolation of crystalline enzyme from fresh latex.

Crystalline mercuripapain is obtained by dissolving crystalline papain in 0.001*M* mercuric chloride in 70 per cent ethanol and allowing the solution to stand in the cold. In mercuripapain, one atom of mercury is combined with 43,500 gm. of enzyme. Since papain has a molecular weight of about 20,700 (128), mercuripapain appears to be a dimer. Mercuripapain is enzymatically inactive, but the activity can be completely restored by treating the dimer with a reducing agent in the presence of a chelating agent.

Chymotrypsinogen.—Hirs (67) reports that chymotrypsinogen can be chromatographed at pH 6 on Amberlite IRC 50 and that this technique can be used to test the homogeneity of the crystalline protein and to prepare chymotrypsinogen of high purity from crude extracts of pancreas.

Protease of Bacillus subtilis.—Methods for culturing the organism to obtain maximal yields of protease in the medium have been investigated by Güntelberg (54). Protease production begins after growth has levelled off. Good yields of protease are accompanied by a simultaneous decrease in the rate of heat production by the culture. Since a crude salt precipitate of the protease consists of one principal electrophoretic component, a straightforward crystallization procedure was developed. It is proposed that this enzyme be named "subtilisin" (55).

Activation

Activation of chymotrypsinogen.—The investigation of the changes which occur during the activation of chymotrypsinogen to the various chymotryp-

[1] The survey of the literature for this review was completed November 1, 1954.

[2] The following abbreviations have been used: DFP for diisopropylfluorophosphate; DP- for diisopropylphosphoryl-; DNFB for 2,4-dinitrofluorobenzene; DNP- for dinitrophenyl-. In naming peptides and peptide derivatives the abbreviations of Brand (18) have been used for amino acid residues: bz for benzoyl; cbz for carbobenzoxy; tosyl for *p*-toluenesulfonyl; OMe for methoxyl; OEt for ethoxyl. The methyl ester and amide of benzoyl arginine become bz.Arg.OMe and bz.Arg.NH_2; TRIS for *tris*-(hydroxymethyl) aminomethane.

sins has been continued, with striking success, by groups in this country and in France.

Gladner & Neurath (46, 47) have determined the C-terminal residues of the chymotrypsinogens and chymotrypsins by treating the proteins with carboxypeptidase in the presence of DFP[2] to inhibit active proteases. Aliquots were withdrawn at various time intervals, and the liberated amino acids were adsorbed on the hydrogen form of Nalcite HCR resin and were eluted with ammonia. Paper chromatography was used for the identification and semiquantitative estimation of the amino acids liberated. For the determination of C-terminal residues, eleven-times recrystallized chymotrypsinogen was passed through a column of Dowex 50 (hydrogen form) to remove remaining peptide impurities. The active enzymes were converted to crystalline DP[2]-derivatives before analysis except in the case of chymotrypsin-B which was used as the amorphous DP-derivative. The results of these determinations are summarized in Table 1.

TABLE I

MOLES OF AMINO ACIDS LIBERATED PER MOLE OF SUBSTRATE BY THE ACTION OF CARBOXYPEPTIDASE*

Amino Acid	α-Chtg	DP-α-Cht	DP-β-Cht	DP-γ-Cht	Chtg-B†	DP-Cht-B†
Leucine	0.03	1.0	1.0	1.0	0.2	0.5
Tyrosine	0.03	1.0	1.0	1.0	0.6	1.0
Glycine	0	0.4	0.2	0.2	0.03	0.08
Serine	0	0.12	0.20	0.19	T	T
Aspartic acid	0	+	0.17	0.15	T	T
Asparagine	0	?	0.09	0.09	0.03	0.05
Phenylalanine	0	0	+	+	T	0.03

* + indicates a quantity too small to be estimated and T very faint traces; ? denotes questionable occurrence; as column headings, Cht is used to designate chymotrypsin and Chtg, chymotrypsinogen.

† Very small amounts of lysine, threonine, alanine, and valine were also detected in chymotrypsinogen-B and in chymotrypsin-B.

Reversible heat denaturation of chymotrypsinogen increased the yield of leucine and tyrosine to one-seventh equivalent per mole. Acid denatured chymotrypsinogen yielded one-sixteenth mole of tyrosine but only traces of leucine. In these cases very small amounts of several other amino acids were also found.

The addition of more carboxypeptidase to a solution of DP-α-chymotrypsin, after a 4-hr. period of hydrolysis, resulted in no increase in free amino acids. If, however, the carboxypeptidase in a 4-hr. hydrolysate was denatured in acid solution, and the free amino acids were removed by dialysis, the addition of a second portion of carboxypeptidase, after readjustment of

the pH of the solution, resulted in further hydrolysis of the protein. In a second 4-hr. period 0.36 moles of each of glycine, serine, and leucine were liberated. When this process was repeated, the third cycle of hydrolysis yielded 0.3 mole of serine and of leucine and 0.09 mole of glycine. It was suggested that this behavior arises from inhibition of carboxypeptidase by tyrosine released in the first hydrolysis cycle, since carboxypeptidase activity, tested against synthetic substrates, is found to be undiminished at the end of the first hydrolysis cycle.

After each cycle of hydrolysis, the sedimentation properties of the modified DP-α-chymotrypsin were determined. Since the observed sedimentation constant is that of the monomer of chymotrypsin and since the modified protein does not dimerize, it appears that at least one of the C-terminal groups is involved in the dimerization process.

Carboxypeptidase treatment of diethylphosphoryl-α-chymotrypsin yields the same amino acids as are found with DP-α-chymotrypsin. Since the modified diethylphosphoryl chymotrypsin is reactivated to the same extent by hydroxylamine as is diethylphosphoryl chymotrypsin (27), it is clear that the C-terminal groups removed by carboxypeptidase are not essential to enzymatic activity.

In view of the results then available from C- and N-terminal analyses of chymotrypsinogen and of α-chymotrypsin, of the kinetic results of Jacobsen (71), of the difference in molecular weight between chymotrypsinogen and chymotrypsin, and of the fact that the isoelectric point of chymotrypsin is lower than that of chymotrypsinogen, Gladner & Neurath (46) propose that the initial action of trypsin in the activation process is to open one bond at an arginine or lysine residue to convert chymotrypsinogen, visualized as a cross-linked cyclic peptide, to π-chymotrypsin. In the presence of a high trypsin concentration, a second bond at a basic amino acid residue is opened. The product of this reaction is δ-chymotrypsin. (According to this hypothesis δ-chymotrypsin should have the same isoelectric point as chymotrypsinogen. This point has not been tested.) In the presence of small quantities of trypsin, π-chymotrypsin autolyzes to α-chymotrypsin. In this process, two peptide bonds, each of the type specific for chymotrypsin, are opened. One hydrolysis is visualized as opening the peptide chain at a second point and the other as resulting in the splitting off of a basic peptide having a molecular weight of about one thousand. The peptide postulated in this scheme has been isolated and found to consist of lysine, arginine, aspartic acid, glutamic acid, serine, glycine, leucine, alanine, and possibly valine (103). The molecular weight and essentially basic nature of this peptide are in remarkably good agreement with the properties postulated for this material.

It has been pointed out (103) that since α-, β-, and γ-chymotrypsin have the same C-terminal and N-terminal amino acids, essentially the same molecular weight and the same substrate specificity, the structural basis for the difference between these enzymes is not apparent.

The N-terminal amino acids of these proteins have been studied by

Rovery, Fabre, & Desnuelle (115). Chymotrypsinogen, treated with DNFB,[1] yields no trace of DNP[2]-amino acids following acid hydrolysis. The same wholly negative result is found when the protein is treated by the phenylisothiocyanate technique. On the other hand, although α-chymotrypsin gives rise to nonstoichiometric quantities of several amino acids by the DNFB method, DP-α-chymotrypsin yields one mole each of isoleucine and alanine and no other amino acid. This difference between α-chymotrypsin and DP-α-chymotrypsin is accounted for, in agreement with Jansen *et al.* (72), on the basis that peptides which are firmly bound to the active enzyme can be removed from the DP-enzyme. It was also found that the N-terminal sequence in one chain of chymotrypsin and in the single chain of trypsin (30) is Ileu.Val. Entirely consistent results were found in each case when the DNFB results were checked by the phenylisothiocyanate method (114).

Rovery & Desnuelle (113) reported preliminary studies on δ-chymotrypsin, and Rovery, Poilroux & Desnuelle (116) isolated crystalline DP-δ-chymotrypsin following the addition of an excess of DFP to the rapid activation system. The single N-terminal sequence, determined by the DNFB method, is isoleucyl valine and the C-terminal amino acid, determined by the carboxypeptidase technique, is leucine. An activation scheme (Fig. 1) modified from the proposals of Gladner & Neurath (46), is proposed.

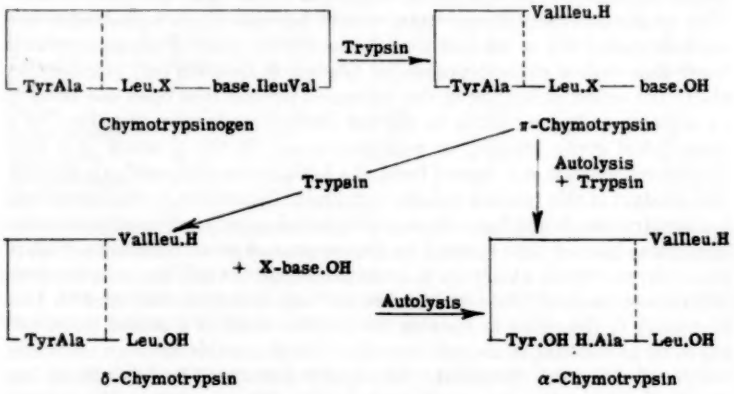


FIG. 1. Activation of chymotrypsinogen.

While this proposal accounts for the information derived from end-group analysis, Jacobsen (71) did not find the autolytic conversion of δ-chymotrypsin to α-chymotrypsin to occur, but did consider the possibility that δ-chymotrypsin might be more resistant to autolysis than are the other forms. Further, in the conversion of π- to δ-chymotrypsin, trypsin is pictured as opening the bond involving the carboxyl of leucine. Since the available evidence indicates that trypsin has the same specificity when it acts on proteins

and high molecular weight peptides that it displays toward synthetic substrates, this postulate appears not to be justified. Chymotrypsin, however, does have activity against norvalyl and norleucyl residues in synthetic substrates (104), and recently it has been indicated that chymotrypsin opens a leucyl bond in corticotropin A (156). It thus seems possible that autolytic steps are involved even in rapid activation.

Activation of trypsinogen.—It has been previously reported by Davie & Neurath (28) that in the native state both trypsinogen and DP-trypsin are resistant to the action of carboxypeptidase, and that a peptide, composed of valine, lysine, and five to six aspartic acid residues, is split off during the activation of trypsinogen. The acidic nature of the peptide is consistent with the isoelectric points of trypsinogen and of DP-trypsin (103). By the DNFB method, the N-terminal amino acids of trypsinogen and of DP-trypsin are valine and isoleucine, respectively [Rovery, Fabre & Desnuelle (115)]. The latter result was checked, with excellent agreement, by the isothiocyanate method.

It has been further found that valine occupies the N-terminal position in the peptide split off during the activation of trypsinogen [Neurath & Davie (101)] and that the N-terminal sequence of trypsinogen is Val $(Asp)_nLys$ or Val $(Asp)_{n-m}.Lys\ (Asp)_m$ [Desnuelle & Fabre (30)] so that there can be little doubt that in the activation of trypsinogen a peptide is hydrolyzed from the N-terminal end of the single available peptide chain. In view of the specificity of trypsin, it is presumed that the C-terminal amino acid in this peptide is lysine (103). Partial acid hydrolysis of the DNP derivative of DP-trypsin reveals the N-terminal sequence of this protein to be Ileu.Val.Gly (30). At present it is not known whether the peptide chain of these proteins is terminated by a cyclic structure or whether the C-terminal residue is resistant to hydrolysis by carboxypeptidase for some other reason. One possibility is suggested by the observation of Acher & Chauvet (1) that the C-terminal group of vasopressin, which is also resistant to attack by carboxypeptidase, is glycinamide.

Pepsin.—The available information on the activation of pepsinogen has been summarized by Herriott (66). Reference is made in this paper to unpublished results indicating that alanine is C-terminal in pepsinogen and pepsin. Williamson & Passman (158) have demonstrated by the carboxypeptidase technique and by the preparation of the DNP derivative of the amino alcohol formed by lithium aluminum hydride reduction that pepsin has two C-terminal alanine residues. Since pepsin has only one N-terminal group, leucine or isoleucine (66), it appears that it may be a branched structure.

Plasmin (fibrinolysin).—According to Bidwell (12), fibrinolysin, in plasma from living subjects, and cadaver lysin may differ only quantitatively but neither is identical with chloroform-activated plasmin. Attempts to demonstrate the existence of a kinase were inconclusive. Müllertz, however, reports (99) that the activity of the plasminogen activator of spontaneously active human blood or of streptokinase-activated human globulin is increased by

di- and tri-carboxylic acids and decreased by diaminomonocarboxylic acids, while the lysis of fibrin clots in the presence of bovine plasmin, trypsin, chymotrypsin, *Aspergillus* protease, or *B. subtilis* protease is not significantly influenced by these compounds. It is concluded that there is a plasminogen activator in spontaneously active human blood which is not plasmin.

From a study of the activation of bovine plasminogen by trypsin, Jackson & Mertz (70) conclude that the action of chloroform in plasminogen activation is to remove an inhibitor. If chloroform treatment is not used, the inhibitor can be overcome by the use of large amounts of trypsin.

General Properties

Chymotrypsin.—Egan, Michel & Schlueter (36) find that seven-times recrystallized chymotrypsin is electrophoretically heterogeneous and that the electrophoretic components can be identified as α-, β-, and γ-chymotrypsin on the basis of mobilities and specific activities. However, when chymotrypsinogen is activated in the presence of DFP, the crystalline product is monophoretic even after prolonged electrophoresis.

Photooxidation of chymotrypsin in the presence of methylene blue results in a rapid and parallel loss of proteolytic and esterolytic activities of the enzyme. [Weil, James, & Buchert (153)]. After four moles of oxygen have been taken up, corresponding to the oxidation of one mole of histidine and three moles of tryptophan, the enzyme is enzymatically inactive and no longer reacts with DFP. When chymotrypsinogen is photooxidized to the extent of four moles of oxygen per mole, it loses the ability to be converted to an active enzyme by trypsin (153). Photooxidized chymotrypsin sediments as a monomer at all protein concentrations [Egan *et al.* (36)]. At pH 4.6 the electrophoretic mobility is decreased 20 per cent and the heterogeneity is increased compared to chymotrypsin itself. When DP-chymotrypsin is photooxidized, the rate and extent of oxygen uptake and of histidine destruction are approximately half those found with the active enzyme, and the electrophoretic mobility of the oxidation product is unchanged relative to that of DP-chymotrypsin.

Wood & Balls (161) observe that when chymotrypsin serves as an acceptor in the peroxidase-hydrogen peroxide system, one mole of oxygen is taken up per mole of enzyme. After oxidation, a crystalline protein, similar in crystal habit to chymotrypsin, can be isolated. This protein, however, has only half the activity toward tyrosine ethyl ester of native chymotrypsin.

McMeekin, Groves & Hipp (94), in a continuation of their studies of the hydration of protein crystals, find the density of DP-α-chymotrypsin crystals to be 1.183. The water content of the crystals is 40.9 per cent and the specific volume of the protein is 0.736, in good agreement with the reported value.

The report (124) that the extent of dimerization of α-chymotrypsin in solution varies with the age of the solution could not be confirmed [Schwert (118)]. In this connection, the observations that dimerization of α-chymotrypsin depends upon intact C-terminal carboxyl groups (47), that δ-chymo-

trypsin contains only one C-terminal carboxyl (113), and that δ-chymotrypsin dimerizes to a smaller extent than does the α-form (120) seem wholly consistent.[3]

McLaren (92) reports the adsorption of proteins on kaolinite to be markedly diminished at pH values alkaline to their isoelectric points. In agreement with this generalization, the adsorption of chymotrypsinogen and of chymotrypsin is dimished when the pH is raised to the range 8 to 10. By this criterion the isoelectric point of trypsin appears to lie at about pH 8. It is also reported (93) that the adsorption of trypsin or chymotrypsin on kaolinite is complete in 2 to 3 min.

Kleczkowski (82) presents data which suggest that ultraviolet irradiation can alter the stability of chymotrypsin without causing a loss of enzymatic activity.

Trypsin.—Sri Ram and co-workers (133) reason that since trypsin hydrolyzes peptide bonds of residues having cationic side chains, the autolysis of trypsin should be diminished by masking the cationic groups of the enzyme by acetylation. In agreement with this reasoning, trypsin, in which 86 per cent of the amino groups are acetylated, is much more stable in alkaline solution than is trypsin itself. Calcium ion stabilizes both acetylated and native trypsin in alkaline solution. In acid solution acetylated trypsin is less stable than trypsin. The isoelectric point of the acetylated product is about 3.8 as compared to a value of 10.8 which has been reported for trypsin. As previously reported by Fraenkel-Conrat *et al.* (44), acetylated trypsin combines with ovomucoid to a much smaller extent than does trypsin.

The discrepancy between the isoelectric points found for trypsin by the microelectrophoretic method and by the moving boundary technique has been assigned by Hartman, Bateman & Edelhoch (64) to the formation of an inactive form of trypsin or of decomposition products of trypsin at the surface of the particles used in the microelectrophoretic method.

Gutfreund & Sturtevant (56) find that when the pH of a trypsin solution is lowered from 2.5 to 1.4, a first-order reaction is initiated which absorbs 8 kcal. per mole of trypsin at 25°C. The reaction is wholly reversible and is not attributable to aggregation of the protein. It is considered probable that the cause of this heat effect may lie in electrostatic repulsions between positive charges on the peptide chain. Similar effects were observed with bovine serum albumin but not with insulin. In this connection, the observation of Linderstrøm-Lang & Schellman that the A chain of insulin appears not to be a folded peptide (87) may be relevant.

The sensitivity of dry trypsin to inactivation by ultraviolet radiation is reported to increase three-fold between 90°K. and room temperature and another six times when the temperature is increased to 450°K [Setlow & Doyle (122)]. McDonald (91) has studied the inactivation of trypsin in aqueous solutions by x-rays.

[3] It is of interest that unpublished results from this laboratory show the dimerization of chymotrypsin to decrease as the pH of the system is decreased below pH 4.

Pepsin.—The problem of the relation of the structure of pepsin to its enzymatic activity appears to have been considerably advanced by the observation by Perlmann (107) that dialyzable, active fragments are formed during the autolysis of pepsin. A 1 per cent solution of crystalline pepsin in acetate buffer, pH 5.6, was incubated at 37°C. for 24 to 48 hr. It was then dialyzed for 24 hr. at 5°C. against acetate buffer. The dialysate contained 10 to 20 per cent of the nitrogen and 1 to 5 per cent of the activity of the original solution. The dialyzable activity was lost following 5 min. heating at 100°C. or prolonged autolysis at 37°C. Passage of the dialysate through 4 per cent cross-linked Dowex 50 separated at least two active components. Although the active material had only 3 per cent of the specific activity of the original solution with respect to hemoglobin as a substrate, it had 64 per cent of the original specific activity toward acetyl-L-phenylalanyldiiodotyrosine.

Flavin (37), using chromatographic methods to separate the peptides in partial hydrolysates of pepsin, finds the single phosphorus of pepsin to appear in the sequence threonylphosphoserylglutamic acid. The acid-catalyzed nitrogen-to-oxygen acyl migration, characteristic of seryl peptides, is abolished by phosphorylation of the serine oxygen. It is of interest that phosphoserine adjacent to a dicarboxylic acid has also been reported for ovalbumin and casein.

Klotz & Loh Ming (84) find that strongly anionic proteins, such as pepsin, bind the neutral dye, pyridine-2-azo-*p*-dimethylaniline, in the presence of divalent metal ions but not in their absence. The metal appears to act as a true mediator between the protein and the dye. The effectiveness of various metals in promoting binding of the dye is in the order of the chelate stability constants for the ions. Carr, using membrane electrodes, could detect no chloride binding by pepsin at pH 3.87 but did find large amounts of calcium to be bound at pH 7.4 (23).

Goldfarb (48) reports that egg albumin, bovine serum albumin, and gelatin form precipitates with pepsin at pH 4.0. Similar findings were reported last year by Yasnoff & Bull (165). Nine kc. sonic vibration has a negligible effect on dilute solutions of crystalline pepsin [Naimark & Mosher (100)].

Papain.—The somewhat confusing literature on the activation of papain has been clarified by Kimmel & Smith (80) who find that both reduction of disulfide bonds and chelation of traces of heavy metal impurities are required for maximal activity.

Smith, Kimmel & Brown (128) find crystalline papain to be essentially monophoretic over the pH range 3.4 to 9.5 although it is noted that boundary spreading at pH 3.4 and 4.4 is greater than would be expected of a homogeneous substance. At pH 2.8 the enzyme is irreversibly inactivated and the electrophoretic pattern indicates marked heterogeneity. The isoelectric point is at pH 8.75. Since the mobility is essentially constant between pH 3.9 and pH 6.0, it is inferred that the pK values of the 16 ionizable carboxyl groups (130) are of the order of 3.0 to 3.4 and that of the single imidazolium group is about 6.8.

The sedimentation behavior of papain was found to be quite complex. In the presence of 0.02 *M* cysteine at pH 3.9 to 4.0, the sedimentation constant was found to be 2.42 S with little dependence on protein concentration. If cysteine were omitted or if the enzyme were slightly oxidized with iodine, the sedimentation constant increased linearly with protein concentration at pH 4.0. Under these circumstances only one boundary was seen and the sedimentation constant, extrapolated to zero protein concentration, was still 2.42 S. If the pH of the system were increased to 5.4, there was an even more marked dependence of sedimentation rate on protein concentration, even in the presence of cysteine. These results are held to indicate the existence of a readily reversible dimerization, such as has been postulated to explain the behavior of the various chymotrypsins and of trypsin, but there appears to be no evidence from the present measurements to indicate dimerization in preference to any other degree of association. At pH 8.0 in the absence of Versene (ethylenediaminetetraäcetic acid tetrasodium salt), two boundaries, having sedimentation rates of about 2.6 S and 6.0 S, were observed. The proportion of the heavier component increased with increasing protein concentration and, since the amount of heavy component was markedly reduced in the presence of Versene, the effect seems to be attributable to traces of heavy metals.

The diffusion constant, measured at pH 3.9 in the presence of 0.02 *M* cysteine, is 10.27 sq. cm. per sec. The molecular weight is found to be 20,700, using a value of 0.724 [calculated from amino acid composition (130)] for partial specific volume. The frictional ratio is 1.16.

The physical properties of crystalline mercuripapain were also investigated (128). At pH 4 mercuripapain is ultracentrifugally monodisperse and, within the limits of the precision of the measurements, appears to be identical in its behavior with papain. Electrophoretic studies on mercuripapain at this pH reveal the presence of two boundaries. One, with an area about 35 per cent of the total, has the mobility of papain; the other migrates at a higher rate and is believed to consist of one molecule of papain combined with one atom of mercury, the residual charge on the mercury increasing the total charge on the protein. The larger, faster-moving boundary disappears when cysteine and Versene are added to the system. Boundaries with sedimentation rates of 2.42 S and 8.05 S are seen in the ultracentrifuge at pH 8.0. The proportion of the heavier component increases rapidly with increasing protein concentration. The heavier component is estimated to be a hexamer in equilibrium with the monomer of mercuripapain. The existence of two resolvable boundaries in the ultracentrifuge would seem to indicate that if this interpretation is correct, the equilibrium must be slowly established.

The amino acid composition of crystalline papain has been studied independently by Close, Moore & Bigwood (25) and by Smith, Stockell & Kimmell (130). The former investigators used a preparation of papain crystallized by the method of Balls, Lineweaver & Thompson (7) from liquid papaya latex, and the latter group used papain crystallized by the procedure developed in their laboratory (*vide supra*). The preparation of Close *et al.* was

shown to be electrophoretically homogeneous at pH 5.5. Most of the results of both groups were derived from chromatography on Dowex-50 (98). Details of other procedures are given in the original papers. Close, Moore & Bigwood used a single hydrolysis time of 20 hr. in 6 *N* HCl. A 95 per cent recovery of nitrogen was achieved, using a nitrogen content of 16.4 per cent, and 88 per cent of the weight of amino acid residues was accounted for. Smith *et al.* used 70 hr. hydrolysates in the same reagent for most of their determinations but corrected their results for the destruction of serine, threonine, glutamic acid, aspartic acid, and lysine by determining these amino acids at various time intervals and extrapolating the values to zero time to obtain an estimate of the content of these amino acids in the absence of destructive processes. While the assumptions underlying this correction procedure are difficult to test, the method seems to work well for both carboxypeptidase (129) and papain. Using a nitrogen content of 16.1 per cent, 103.8 per cent of the nitrogen and 98.6 per cent of the residue weight were accounted for.

In general the agreement between the two sets of data is good. The exceptions are: (*a*) Close *et al.* find 1.82 gm. of cystine residue per 100 gm. of protein when cystine is estimated as cysteic acid while Smith *et al.* find 3.89 gm. of this residue by assigning the complete sulfur content of papain to this amino acid. The justification for this procedure is that there is no methionine in the protein. (*b*) With the exception of cystine and histidine the values found by Smith *et al.* are higher than those found by Close *et al.* If the values found for cystine are neglected, the average ratio of the results of Smith *et al.* to those of Close *et al.* is 1.09. If the values for serine, threonine, etc., for the destruction of which Smith *et al.* made special corrections, are omitted from consideration, the mean ratio of the results of the two groups of investigators is 1.08. This result suggests the possibility that the error of the two groups in estimating individual amino acids may have been less than the error of estimation of total nitrogen and moisture in the protein samples.

It is clear from both sets of data that there is no methionine in papain and only one histidine residue per mole. Tyrosine, on the other hand, occurs in unusually high proportion. Smith, Stockell & Kimmel calculate an average molecular weight of 20,340 from all the amino acid values, and the same molecular weight is found if only the values for amino acids occurring to an extent of less than ten residues per mole are used. There is a total of 178 residues and 19 cationic and 16 anionic groups, a finding which is consistent with the high isoelectric point of the enzyme.

Thompson (138), using the DNFB technique, has found the single N-terminal amino acid of papain to be isoleucine and reports the N-terminal sequence to be Ileu.Pro.Glu. Since longer peptides are not found in partial acid hydrolysates, it is considered possible that the next residue is serine, threonine, or tryptophan.

Subtilisin.—Crystalline subtilisin has been found by Güntelberg & Ottesen (55) to be monophoretic at pH 5.3 and 6.5, but it exhibits pronounced boundary spreading, probably arising from autolysis, at pH 8.1 and

9.5. The crystalline enzyme attacks a variety of proteins, has no amylase activity, but does hydrolyze methyl butyrate. [The true esterase activity of the crude enzyme has been studied by Graae (51)]. The pH optimum, with casein as substrate, is 10.0 at 35.5°C. Subtilisin attacks casein more rapidly and carries hydrolysis further than does trypsin but is slightly less active than trypsin when hemoglobin is the substrate. Further, subtilisin is more stable than trypsin in alkaline solution but is rapidly inactivated at pH values below 5. Subtilisin is not inhibited by Versene, cyanide, iodoacetate, or mercuric chloride but is 90 per cent inhibited by $10^{-4}M$ DFP in 30 min.

Ottesen & Wollenberger (105) have demonstrated by the carboxypeptidase technique that alanine is the C-terminal amino acid in the hexapeptide which is released in the subtilisin-catalyzed conversion of ovalbumin to plakalbumin.

Plasmin.—It seems likely that the confusing literature describing the enzymes involved in blood clotting and clot lysis may be clarified by the observation of Troll, Sherry & Wachman (143) that plasmin contains two enzymes having different specificities toward synthetic substrates. Streptokinase-activated plasmin hydrolyzes bz.Arg.OMe,[3] Lys.OEt, and the methyl, ethyl, and butyl esters of tosyl.Arg. Although the hydrolysis of a variety of other amino acid esters was not found to be potentiated by streptokinase, the hydrolysis of the susceptible substrates was markedly increased by streptokinase activation. The pH optimum for the hydrolysis of tosyl. Arg.OMe is pH 9 while that for Lys.OEt is pH 6.5. This effect may be the result of transpeptidation on the unsubstituted α-amino of lysine. As might be expected, bz. Arg. NH_2 is hydrolyzed at a much lower rate than is bz. Arg. OEt. Heating plasminogen to 50°C. for 30 min. at pH 7 caused a rapid loss of potential activity towards Lys. OEt but no loss of potential tosyl. Arg. OMe activity. All reported procedures for the preparation of plasminogen give rise to mixtures of these two enzymes [Troll (142)].

Sherry & Troll (123) further report that thrombin attacks bz.Arg.OEt, tosyl.Arg.OMe, Arg.OMe, and bz.Arg.NH_2 but not Lys.OEt, ac.Tyr.OEt, or ac.Leu.OEt. The pH optimum for the hydrolysis of tosyl.Arg.OMe is 9.0 in TRIS[3] buffer, 8.0 in borate, and 7.6 in phosphate. Since the thrombin preparation had a high clotting activity, no activity toward Lys. OEt, and little activity with respect to casein, these results are not considered to arise from plasmin present as a contaminant. Soybean trypsin inhibitor partially inhibits plasmin but does not inhibit thrombin. The activation of whole plasma with thromboplastin and calcium ion causes a marked increase in activity toward tosyl.Arg.OMe. Further, the ratio of this activity to clotting activity is essentially constant in three preparations of thrombin, and the two activities are removed in parallel fashion by adsorption on various gels. The ester substrate inhibits clotting, and the inhibition disappears as the substrate is hydrolyzed. While these results clearly indicate the activity of thrombin on tosyl.Arg.OMe to be attributable to thrombin itself, the possibility that the tosyl.Arg.OMe activity of plasmin is attributable to throm-

bin seems not to have been eliminated. Since Kleinfeld & Habif (83) have shown prothrombin, like plasminogen, to be activated by trypsin, this possibility does not seem completely remote.

Carboxypeptidase.—The problem of the possible existence of carboxypeptidase as a metal-activated enzyme seems to have been clarified during the past year. It was inferred by Smith & Hanson (126, 127) on the basis of inhibition by various anions and on the qualitative detection by emission spectroscopy of magnesium in the crystalline enzyme, that carboxypeptidase is a magnesium peptidase. This finding was questioned by Neurath & DeMaria (102) who found the initial reaction velocity not to be influenced by orthophosphate, citrate, oxalate, cyanide, or pyrophosphate but did find that these ions promoted inhibition by reaction products.

Vallee & Neurath (146) now report that by quantitative emission spectrography and by the use of diphenylthiocarbazone as a colorimetric reagent, 0.96 moles of zinc are found in one mole of crystalline carboxypeptidase (34,300 gm.). All other alkali and alkaline earth metals, including those used in the preparation of the enzyme, are absent or present in stoichiometrically negligible quantity. Activity and zinc content increased in parallel fashion during the purification of the enzyme. The metallic ion could be removed by chelating agents but not by dialysis against water or ammonia solution.

Another effect of ions on carboxypeptidase has been studied by Gorini & Labouesse-Mercouroff (49) who report that at low ionic strengths divalent cations activate the enzyme more than do monovalent cations, but that at high ionic strengths this difference disappears. These investigators also found Tween 20 and Tween 80 to be very effective activators for the enzyme, 0.005 per cent Tween 20 in 0.02M NaCl bringing the enzyme to a relative activity of 142 as compared to a relative activity of 100 in 0.62M NaCl. It was further found that at low ionic strengths enzyme activity is not a linear function of carboxypeptidase activity, low concentrations of the enzyme being less active than higher concentrations. This anomaly disappeared when the ionic strength was increased or when Tween was added to the system. Since neither salts nor detergents protect the enzyme against heat inactivation, it was concluded that these substances act to prevent the formation of inactive intramolecular complexes by the enzyme.

Smith & Stockell (129) have carried out a complete amino acid analysis of crystalline carboxypeptidase by the Dowex 50 method of Moore & Stein (98). Although all the usual amino acids are found in carboxypeptidase, it is distinguished by an unusually high content of serine and threonine, 33 and 27 residues per mole, respectively, and by a single methionine residue. A total of 310 residues was found. An average molecular weight of 34,360 was calculated using the values found for all the amino acids, and a molecular weight of 34,440 was calculated from the content of the five amino acids occurring in smallest amounts. The agreement with the values previously found by physical methods, 31,600 and 32,000 (109,129), is clearly excellent.

In a study of the thiohydantoin method for determining the N-terminal

sequence of proteins, Thompson (139) found the N-terminal residue of carboxypeptidase to be asparagine. This confirms his previous finding, by the DNFB technique, that the N-terminal sequence is asparaginylserine (137).

Inhibition by Acyl Phosphates

Chymotrypsin.—Schaffer, Harshman & Engle (117) report the inversion of phosphoserylglycine during hydrolysis in 2*N* HCl at 100°C. However, since hydrolysis of DP-chymotrypsin with 12*N* HCl at 37°C. yields phosphoseryl glycine, rather than glycylserine phosphoric acid, it is concluded that the former is probably the normal sequence in the inhibited enzyme.

Hartley & Kilby (62) have previously observed that chymotrypsin, which has been completely inhibited toward protein, ester, and amide substrates with diethyl *p*-nitrophenyl phosphate, retains some degree of hydrolytic activity toward *p*-nitrophenyl ethyl carbonate and *p*-nitrophenyl acetate. It has now been found that insulin also catalyzes the hydrolysis of the *p*-nitrophenyl esters (63). Since Phe.OEt reacts stoichiometrically with *p*-nitrophenyl acetate to yield ac.Phe.OEt, it is concluded that the hydrolytic action of proteins involves the formation of an acetylated protein which then hydrolyzes spontaneously. The Arrhenius activation energy for the reaction of chymotrypsin with diethyl *p*-nitrophenyl phosphate is reported by Kilby & Youatt (79) to be 21.6 kcal. per mole and that for the reaction with O,S-diethyl-O-*p*-nitrophenyl phosphorothiolate to be 11.3 kcal. per mole.

Cunningham (27) finds that the reactivation of diethyl-*p*-nitrophenyl-phosphate-inhibited chymotrypsin, by hydroxylamine, can be formally described by first-order kinetics at constant hydroxylamine concentration. However, the rate constant is found to increase with increasing hydroxylamine concentration even though hydroxylamine is present in great excess in all cases. The Arrhenius activation energy is 11.4 kcal. per mole at constant hydroxylamine concentration at pH 7. In no case could more than 50 per cent reactivation be obtained. The effect of pH on the reactivation could be described by assuming that only the free base of hydroxylamine is reactive and that the dissociation of a proton from the inactive enzyme at a pH near 8 renders it resistant to reactivation. In agreement with Wilson *et al.* (160), reactivation is regarded as a displacement of the phosphate diester from the enzyme with the concomitant formation of a phosphohydroxamic acid which is rapidly and spontaneously hydrolyzed.

Trypsin.—The inhibition of trypsin by *p*-nitrophenyl-diethylphosphate and by O,S-diethyl-O-*p*-nitrophenyl phosphorothiolate has been found to be stoichiometric [Kilby & Youatt (79)]. The reaction is first-order in the presence of an excess of inhibitor and is characterized by an Arrhenius activation energy of 15.5 kcal. per mole for the acyl phosphate and of 13.0 kcal. per mole for the phosphorothiolate. As has also been observed for chymotrypsin, both *p*-nitrophenyl acetate and *p*-nitrophenyl ethyl carbonate are hydrolyzed catalytically by trypsin.

Inhibition by Naturally-Occurring Inhibitors

Chymotrypsin.—Wu & Laskowski (163) have found that although various naturally-occurring trypsin inhibitors also inhibit α- and β-chymotrypsin, none forms a stoichiometric complex with either chymotrypsin. The differences in the activity of some inhibitors toward α- and β-chymotrypsin is taken to indicate a structural difference between the two chymotrypsins. Neither the pancreatic inhibitor of Kazal *et al.* (77) nor ovomucoid inhibited either chymotrypsin.

Melchoir & Sliwinski (96) find the inhibition of trypsin by the inhibitors in rat serum to be a relatively slow process compared to the rate of inhibition of chymotrypsin. Since the level of inhibitor is lower in the serum of weanling rats than in that of adult rats and is not affected by severe fasting but does fall following hypophysectomy, it is concluded that serum inhibitor levels are under endocrine control.

Trypsin.—Sri Ram *et al.* (132) find from ultracentrifugal measurements that the trypsin-ovomucoid complex is stable between pH 4.7 and 7.7 and is wholly dissociated at pH 2.7. In the presence of excess ovomucoid, two boundaries, having the sedimentation characteristics of the complex and of ovomucoid, are detected. However, in the presence of excess trypsin, only a single boundary is seen. As the concentration of trypsin is increased, the sedimentation rate of the single boundary progressively decreases from the value characteristic of the complex. In electrophoretic studies the trypsin-ovomucoid complex again behaves as an independent entity. The mobility of the complex is very low over the pH range from 5 to 10. This observation is compatible with the hypothesis that the formation of the complex involves a mutual neutralization of electrostatic charges. In the presence of excess trypsin, three electrophoretic components are seen. Of these, one has the characteristic mobility of trypsin, one is the trypsin-ovomucoid complex, and the third is believed to be the complex of two molecules of trypsin with one of ovomucoid. The dissociation constant of the trypsin-ovomucoid complex was found to be 5.8×10^{-9} M in good agreement with the value found by Green (52). The dissociation constant for the dissociation of trypsin from the $(\text{trypsin})_2$-ovomucoid complex was estimated to be 2.5×10^{-6}M.

Green (52) finds the inhibition of trypsin by DFP, by ovomucoid, and by soybean and pancreatic trypsin inhibitors to be of the "competitive" type since bz.Arg.OEt prevents the reaction of these inhibitors with trypsin. Further, DFP reacts to a negligible extent with the trypsin-pancreatic inhibitor compound and DP-trypsin does not combine to an appreciable extent with any of the protein inhibitors. On this point there is an apparent contradiction between these results and an abstract report of work by Jansen, Jang & Olcott (73) which states that DP-trypsin does combine with natural inhibitors although the combination is weaker than with trypsin. Jansen *et al.* also report the isolation of a crystalline DP-trypsin-soybean inhibitor complex.

Green (52) further finds that calcium ion, a nonessential activator (53),

does not influence the amount of soybean or pancreatic inhibitor required to produce a given degree of inhibition nor does it influence the rate of hydrolysis of bz.Arg.OEt in the presence of DFP nor the rate of reaction between trypsin and DFP. The latter findings were taken to indicate that DFP reacts with both the active and inactive forms of trypsin. It was also found that while bz.Arg.OEt does not prevent the inhibition of trypsin by $10^{-4}M$ mercuric chloride, mercuric chloride does prevent the reaction of DFP and pancreatic inhibitor with trypsin. It is concluded that since DFP and pancreatic inhibitor compete with bz.Arg.OEt and also with mercuric chloride, mercuric chloride must also compete with the substrate. Although Green considers and rejects the suggestion that a new term, such as "interference" (2), should be used to describe inhibition by protein inhibitors or DFP, it may be pointed out that the available data are consistent with the view that the protein inhibitors are bound to trypsin at sites which are probably not involved in the combination of the enzyme with its substrates and that DFP acts by forming a different type of bond than is involved in the enzyme-substrate complex. If wholly different forces are involved in binding substrate and in binding inhibitor, the term "competitive" is difficult to understand. In the case of the protein inhibitors and bz.Arg.OEt mutual prevention of reaction, rather than competition for the active center, seems to occur.

An unexpected effect of sequence of mixing of the reactants in trypsin-inhibitor-substrate systems has been found by Viswanatha & Liener (150). With soybean inhibitor, Kunitz' pancreatic inhibitor, ovomucoid, and the inhibitor of Kazal *et al.* (77), 1.3 to 2.2 times greater inhibition of trypsin was observed when a solution of substrate (casein) and inhibitor was added to the enzyme than when a solution of enzyme and inhibitor was added to the substrate solution. The same effect is observed when denaturation of β-lactoglobulin by trypsin is the measured variable. In the case of soybean inhibitor, effects attributable to sequence of mixing were not observed at pH 4.2 or 5.6 but were found at pH 7.6. The sequence-of-mixing effect disappeared in the presence of $0.01M$ calcium. The subsequent addition of sequestering agents counteracted the activating effect of calcium but did not cause the sequence-of-mixing effect to reappear. It is considered that the simultaneous addition of substrate and inhibitor to the enzyme solution causes combination of the inhibitor with some polymeric form of trypsin, which presumably dissociates in the presence of inhibitor alone. In view of Green's observations (52), it appears equally possible that in the presence of substrate the inactive form does not revert to the active form, so that only enough inhibitor need be added to combine with the active form of trypsin. It was also observed that the trypsin-lima bean inhibitor and acetylated trypsin-soybean inhibitor systems exhibit no sequence-of-mixing effects. While this observation was interpreted as a case of uncompetitive inhibition, the premise being that the inhibitor can combine only with the enzyme-substrate complex, it seems possible that the inhibitor combines with both the inactive and the active forms of trypsin.

Steiner (134) has studied the association of soybean inhibitor with trypsin by means of light-scattering and fluorescence polarization techniques. The molecular weight of trypsin is found to be 24,000 and that of the inhibitor 20,000. A consistent molecular weight of 44,000 was found for the trypsin-inhibitor complex. In essential agreement with studies by other techniques, dissociation of the complex occurs when the pH is lowered from 4.4 to 3.3. Dissociation also increases with increasing ionic strength of the medium. These observations are compatible with the view that association results from interaction of specific groups of opposite charge on the enzyme and inhibitor molecules. The apparent entropy change on association is found to be an increase of 15 to 20 e.u.

Soybean trypsin inhibitor is reported to have aspartic acid or asparagine as the N-terminal residue and leucine as the C-terminal residue [Neurath & Davie (101)]. The terminal carboxyl is apparently not involved in the combination with trypsin since crystalline trypsin-inhibitor complex yields only leucine as the C-terminal acid. Moreover, after removal of the C-terminal leucine from the soybean inhibitor by the action of carboxypeptidase, the inhibitor has undiminished activity toward trypsin.

A number of reports have appeared which indicate possible future fields of activity with respect to the naturally-occurring inhibitors. The ovomucoid of duck egg white has been found to be a powerful trypsin inhibitor [Vijayaraghavan & Narasinga Rao (149)]; the proteolytic activity of snake and spider venom, like that of trypsin, can be inhibited by human serum, and the concentrations of serum necessary to inhibit the three activities are of the same order [Kaiser (76)]. Bowman (17) finds that soybean and navy-bean trypsin inhibitors reduce the incidence of fatal trypsin embolism when they are injected intravenously into rabbits with trypsin. Thromboplastic activity is retained, however, by the trypsin-inhibitor complex. An improved method for the preparation of the navy-bean inhibitor is given (17).

Activity on Synthetic Substrates

Chymotrypsin.—In a logical extension of the principle that the nature of the hydrolyzable group is not critical for chymotrypsin substrates as long as other structural requirements are satisfied, Doherty & Thomas (32) have made the startling discovery that carbon-carbon bonds in ethyl 5-phenyl-3-ketovalerate and ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate are hydrolyzed in the presence of chymotrypsin to ethyl acetate and the corresponding phenyl- or *p*-hydroxyphenyl-propionic acid.

Jennings & Niemann (74) find the Michaelis constant and the rate of decomposition of the enzyme-substrate complex to be essentially the same for the chymotryptic hydrolysis of acetyl-L-hexahydrophenylalaninamide and for the hydrolysis of acetyl-L-phenylalaninamide. This result is taken to indicate that aromatic character is not an essential structural feature of substrates for chymotrypsin and that the cyclic portions of these substrates are

held to the enzyme surface by van der Waals' forces rather than by *pi* electron interactions. Since the hexahydrophenylalanine substrate presumably consists of a mixture of chair and boat forms, the interpretation of kinetic results obtained with this substrate must be attended by some uncertainty. In view of the steric differences between the substrates compared, it seems equally possible to conclude that the cyclic portions of these substrates do not interact with the surface at all but serve to orient the substrate on the enzyme surface only by virtue of their space-filling properties.

Foster, Jennings & Niemann (40) have determined the kinetic constants for the chymotryptic hydrolysis of tyrosinhydroxamide and have found D-tyrosinhydroxamide to be a competitive inhibitor for chymotrypsin. The origin of the pH optima shown by acetyltyrosinamide, acetyltyrosinhydroxamide, and tyrosinhydroxamide, 7.9, 7.6, and 6.9, respectively, has also been considered. It is assumed that the arrangement of charges on the enzyme surface is optimal at pH 7.9, the optimum pH for uncharged substrates. It follows that the lower pH optimum observed with acetyltyrosinhydroxamide results from a decreased concentration of uncharged form of the substrate at pH 7.9, the hydroxamide-hydroxamate dissociation increasing with increasing pH. The still lower pH optimum for tyrosinhydroxamide is accounted for, in accordance with the mechanism proposed by Swain & Brown (135), on the basis that molecules having charged α-ammonium groups are more reactive and that the concentration of this form increases as the pH is lowered. It seems more probable that the reduction in apparent reaction velocity at higher pH values is attributable to transpeptidation onto the uncharged α-amino group (75).

Clark-Lewis & Fruton (24) find γ-Glu.Phe.NH_2 and γ-Glu.Tyr.NH_2 to be hydrolyzed by chymotrypsin. The rate of hydrolysis of γ-Glu.Phe.NH_2, however, is only about one-eighth that of Gly.Phe.NH_2.

Bender, Ginger & Kemp (9) have found that when the chymotrypsin-catalyzed hydrolysis of methyl-β-phenylpropionate, labelled with O^{18} in the carbonyl position, is carried to 50 per cent hydrolysis and the unreacted ester is isolated, no interchange of oxygen between the ester and the water of the medium can be detected. This observation and the related observation that, in the presence of chymotrypsin, oxygen interchange does occur between water and the carboxyl groups of specific amino acids [Sprinson & Rittenberg (131); Doherty & Vaslow (33)] appear to be entirely consistent with the concept of an acyl-enzyme as the active intermediate in chymotryptic reactions.

Trypsin.—The proteolytic coefficients for the hydrolysis of Arg.Leu, Arg.Gly, Arg.Phe, and Arg.Glu by trypsin are 7,3,3, and 2 per cent, respectively, of the value for bz. Arg.NH_2. If the amino group is masked by carbobenzoxylation and the guanidinium group by nitration, these substrates become inactive [Van Orden & Smith (147)].

Papain.—It is reported by Kimmel & Smith (80) that the hydrolysis of bz. Arg.NH_2 follows first-order kinetics. Since adherence to first-order kinetics is usually fortuitous in reactions of this type, it seems possible that, as was

shown for trypsin acting on bz.Arg.NH_2 (60, 119), apparent first-order kinetics may result from progressive inhibition by benzoyl arginine. Papain was also shown to have an esterase activity toward tosyl.Arg.OMe. Two points are of interest in this connection: (*a*) unlike the pH-activity curve for bz. Arg.NH_2 which is flat between pH 5 and pH 7.5, the curve for tosyl. Arg.OMe shows a sharp optimum at pH 6.0; and (*b*) bz. Arg.NH_2 is hydrolyzed about 10 per cent faster than is tosyl.Arg.OMe. Since tosyl.Arg.OMe is hydrolyzed about eight times as fast as bz.Arg.OMe by trypsin (121), it is possible that, in the case of papain, amide substrates may be found to be more sensitive to hydrolysis than ester substrates. An alternate interpretation of these results is that different enzymes, present in crystalline papain, are responsible for the hydrolysis of bz.Arg.NH_2 and of tosyl.Arg.OMe. This possibility appears to be ruled out by the observation of McDonald & Balls (90) that an increase in esterolytic activity accompanies purification of the protease and that esterolytic activity is inhibited by *p*-chloromercuribenzoate and restored by treatment of the inhibited enzyme with cysteine. A low order of esterolytic activity against *p*-nitrobenzoyl-L-arginine methyl ester and against hippuryl methyl ester was also observed.

Kimmel & Smith (80) further report that, while bz.Arg.NH_2 is the most active synthetic substrate tested, a wide variety of compounds is split at lower rates. Other substrates having proteolytic coefficients greater than 1 per cent of that of bz.Arg.NH_2 are cbz. glutamic acid diamide, cbz.isoglutamine, cbz.Leu.NH_2, and bz.Gly.NH_2. The pH-activity curves for these substrates show great dissimilarities. This might be taken to indicate more than one type of enzymatic activity had it not been found that cbz.Glu.OH is a powerful inhibitor for the hydrolysis of all of these substrates by papain.

Van Orden & Smith (147) find that papain has no activity on arginyl dipeptides such as Arg.Leu, Arg.Gly, Arg.Phe or Arg.Glu. However, when the guanidinium group is masked by nitration and the amino group by carbobenzoxylation, these peptides are split at about 0.3 to 1 per cent of the rate of hydrolysis of bz.Arg.NH_2.

Carboxypeptidase.—Yanari & Mitz (164) have studied the hydrolysis of dipeptides by carboxypeptidase and report that Leu.Tyr, which is hydrolyzed at about 1/200 the rate of cbz.Gly.Phe, is a more active substrate than Gly. Tyr, Gly.Phe, Gly.Leu, or Ser.Tyr. Despite the relatively low rates of hydrolysis of these peptides, which are consistent with earlier observations on the dipeptidase activity of this enzyme (11, 68), they may be significant in studies of the amino acid sequence of peptides and proteins since the enzyme is frequently used in relatively high concentrations in such studies. Other limitations of the method were pointed out in last year's review (29). The optical configuration of the amino acid contributing the carbonyl to the susceptible bond was shown to be of great significance only when the amino group of that amino acid is substituted, since D-Leu-L-Tyr was found to be hydrolyzed at nearly the same rate as Leu.Tyr. As anticipated from earlier studies (59), cbz-D-Leu-L-Tyr was found to be resistant to hydrolysis. A clue to the speci-

ficity of carboxypeptidase as a dipeptidase seems to be provided by the observation that under conditions resulting in complete hydrolysis of Arg.Tyr, Ser.Tyr is about 25 per cent hydrolyzed and Glu.Phe is not hydrolyzed at all [White & Landmann (83)].

Wood & Roberts (162) report that in the action of carboxypeptidase on substrates in which the terminal amino acid is N^{15}-labelled glycine, some isotope is incorporated into the protein, some into peptides formed by degradation of the protein during the reaction, and some into the acyl amino acid formed as the second reaction product. It seems to the reviewer that such a revolutionary view must be supported by extraordinarily clear evidence. In the present case, the methods used to fractionate the reaction components for isotopic analysis do not seem to meet this requirement.

Other proteases and peptidases.—Cathepsin C is found not to hydrolyze the γ-glutamyl derivatives of Phe.NH_2, Tyr.NH_2, or Gly.NH_2. It is concluded that the secondary peptide bond must involve the α-carboxyl of an amino acid [Clark-Lewis & Fruton (24)]. Kirshner & Pritham (81) find that cathepsin III (leucinaminopeptidase) hydrolyzes the leucyl derivatives of glycine, β-alanine, and γ-aminobutyric acid at rates of the same order but hydrolyzes Leu.NH_2 at a somewhat higher rate. It appears that steric effects rather than changes in the polarity of the substrates are responsible for these differences. Patterson & Lang (106) present evidence to indicate the presence of more than one leucinaminopeptidase in the salivary gland of *Drosophila*.

Adams, Davis & Smith (3) find that the prolidase-catalyzed hydrolysis of glycylallohydroxyproline is two to five times faster than that of glycylhydroxyproline while that of glycylmethoxyproline is much slower. Further glycylmethoxyproline does not interfere with the hydrolysis of Gly.Pro. It is concluded that the *trans* position of the hydroxyl group relative to the carboxyl in hydroxyproline interferes with the binding of this residue to the enzyme surface.

An enzyme found in extracts of acetone powder of swine kidney hydrolyzes bz.Arg.NH_2 and Arg.Leu at approximately the same rate. This enzyme is not activated by manganese or cysteine [Van Orden & Smith (147)]. Izuyima & Greenstein (69) report that renal aminopeptidase hydrolyzes both isomers of cystinylcystine at approximately the same rate while L-cystinyl-D-cystine and D-cystinyl-L-cystine are hydrolyzed at much lower rates. Renal acylase I acts slowly on the L-L forms and not at all on the L-D or D-L forms. Fones & Lee (39) have studied the action of acylase I against a large series of trifluoroacetyl amino acids. L-leucine is reported to inhibit D-leucylglycine dipeptidase activity in normal tissues but to activate this activity in tumor tissues [Vescia, Albano & Iacono (148)]. The stepwise hydrolysis of diglycyl-3,5-diaminobenzoic acid by autolysates of kidney, pancreas, and intestine has been studied by Akamatsu & Furiya (4). The peptidases of normal serum have been investigated by Fleisher (38). Berg (10) finds no significant difference in Ala.Gly peptidase activity between unfertilized eggs and first cleavage blastomeres of *Mytilus edulis*.

Activity on Naturally-Occurring Substrates

Chymotrypsin.—Gergely, Gouvea & Karibian (45) find that chymotrypsin produces two fragments from myosin which are indistinguishable from those produced by the previously investigated action of trypsin on myosin with the single exception that the light fragment (M~100,000) produced by chymotrypsin cannot be crystallized at low ionic strengths.

Under a variety of conditions, chymotryptic hydrolysis of crystalline horse serum albumin yields not less than four electrophoretically-resolvable components. Dialysis of the hydrolysates prior to electrophoresis reduces the number of components to three. It is considered that these nondialyzable fragments are portions of the albumin molecule which resist the action of chymotrypsin [Biserte & Boulanger (13)].

White & Landmann (157) have identified Glu.Phe, Arg.Tyr, and Ser.Tyr in chymotryptic hydrolysates of corticotropin-A. It is concluded from a reconstruction of a portion of the peptide chain of this hormone that chymotrypsin opens a Leu.Glu bond but fails to open a phenylalanyl bond. The bond resistant to chymotryptic hydrolysis was shown by White (156) to be the Phe.Pro bond. It is possible that this result arises from the steric effect of the proline residue.

Trurnit (144) has used the recording ellipsometer to study the interaction of chymotrypsin with layers of bovine serum albumin deposited on barium stearate coated slides. In very dilute Veronal buffers the principal reaction is adsorption of the enzyme on the serum albumin surface. In 0.025*M* to 0.1*M* Veronal buffers there is an adsorption period, which decreases with increasing buffer concentration, followed by a progressive decrease in the thickness of the albumin deposit. The velocity of decrease in thickness increases linearly with the number of layers of serum albumin deposited between 4 and 10 layers and is independent of the orientation of the substrate layers. The Arrhenius activation energy for the surface reaction is 8 to 9 kcal. per mole as opposed to a value estimated at 18 kcal. per mole for the reaction in solution.

Trypsin.—The demonstration by Sanger that trypsin exhibits the same specificity toward large peptides that has been established for synthetic substrates has led to a number of uses of trypsin as an analytical tool. Acher & Chauvet (1) confirm the finding of du Vigneaud, Lawler & Popenoe (35) that trypsin liberates glycinamide from vasopressin. Monier & Jutisz (97) find tryptic hydrolysis of DNP-salmine to yield DNP.Pro.Arg and DNP. Pro.Arg-Arg. Further, if salmine is hydrolyzed by trypsin until 18 to 19 bonds have been opened and the resulting peptides are converted into DNP derivatives and fractionated by countercurrent distribution and column chromatography, the following peptides can be identified: Ileu.Arg, Val.Ser.Arg, Pro.Arg, Ala.Ser.Arg, Gly.Gly.Arg, Ser.Ser.Arg, and arginine itself. It should be noted that it is the occurrence of arginine in every peptide which provides confirmatory evidence for the specificity of trypsin rather than the structures

shown since it was assumed in the structure determinations that trypsin opens the bond at the carboxyl of arginine.

A further example of the activity of trypsin in converting one biologically active protein to another active form is provided by the observation that trypsin, preceding more general proteolysis, acts upon phosphorylase-*a* to convert it to phosphorylase-*b* in the same way as does the prosthetic removing enzyme [Keller (78)].

Halliwell (58) reports the isolation of a new, highly active form of crystalline type-A botulinus toxin. Papain has no effect on the new form, pepsin has a slight detoxifying effect, and trypsin markedly reduces toxicity with a very small accompanying increase in ninhydrin-reactive material. More general hydrolysis appears to follow the loss of toxicity.

Wills (159) finds that 0.001 *M* sodium dodecyl sulfate has no effect on the hydrolysis of casein by trypsin but that the reaction is inhibited by 0.01 *M* concentration of this detergent. On the other hand, 0.01 *M* sodium dodecyl sulfate accelerates the action of trypsin on native and heat-denatured horse serum and on native horse serum albumin, ovalbumin, and fibrin. No effect was shown on the heat-denatured forms of the latter proteins. It appears that the action of the detergent is to denature the protein substrates.

Talwar, Macheboeuf & Basset (136) find that at a pressure of 500 atmospheres the rate of hydrolysis of proteins by trypsin is increased but that at 6000 atm. the reaction velocity is decreased. At a pressure of 500 atm., in the presence of excess denatured crystalline horse serum albumin as substrate, the volume change acompanying reaction is −13.7 ml. per mole of bonds split. However, if the substrate is present in a concentration sufficient to give about half the maximum reaction velocity, the volume change accompanying reaction is only −2.9 ml. per mole.

Pepsin.—White's brilliant accomplishment in determining the C-terminal sequence of corticotropin-A (156) leads to the conclusion that pepsin opens Leu.Ala, Phe.Pro, and Glu.Phe bonds in this sequence.

There is a marked difference in the action of pepsin and trypsin in the hydrolysis of iodinated globin, insulin, and casein, in that pepsin liberates iodinated amino acids from these artificially iodinated proteins much more readily than does trypsin. In the case of thyroglobulin, however, both enzymes release the various iodinated amino acids. The lability of thyroglobulin in this respect is regarded as essential to its physiological function [Roche *et al.* (111)].

The general observation that specificity is greatest when the environment is unfavorable to hydrolysis seems to be verified by the observation of Biserte, Boulanger & Dautrevaux (14) that the hydrolysis of crystalline horse serum albumin by pepsin consists of discrete steps under conditions favoring limited hydrolysis but that the stepwise character of the process disappears under conditions favoring general hydrolysis.

Biserte & Pigache (15) studied the relative rates of solubilization of the sulfur-containing amino acids and of tyrosine when wool or reduced wool is

acted upon by pepsin and when wool or oxidized wool is hydrolyzed in dilute sulfuric acid. The results are consistent with a model of wool keratin in which peptide chains having a relatively high content of tyrosine at the ends of the chains are held together by disulfide bridges which are relatively more concentrated at the center than at the ends of the chains.

Heinicke (65) finds confirmation of the view that the clotting of milk is a stepwise process, in the observation that below 14°C. rennin, pepsin, and trypsin clot milk very slowly while rennin or pepsin together with trypsin causes rapid clotting.

Other proteases.—An enzyme found in extracts of rat liver hydrolyzes insulin but does not attack lactalbumin, serum albumin, or casein [Tomizawa *et al.* (141)]. On the other hand, it appears that the prolongation of the effect of protamine-insulin depends upon the rate of hydrolysis of protamine by an enzyme found in the thrombin fraction of serum. Since this protaminase is activated by streptokinase, it is probably identical with plasmin. Subcutaneous tissue contains a protaminase which is not streptokinase-activated [Brunfeldt & Poulson (19)].

Buchs (21) has presented a polemical summary of his arguments for the existence of gastric cathepsin and has reported that similar evidence for the existence of a cathepsin as well as pepsin can be found from extracts of portions of the entire digestive tract (22). Tolckmitt (140), however, finds pronounced anion effects on the proteolytic activity of gastric juice and ascribes the two maxima in pH-activity curves, observed by Buchs, to these effects. The peptidases and protease of thyroid extracts have been investigated by Weiss (154).

Martin (95) has detected three proteolytic enzymes in extracts of acetone powder of rat skin. It is reported by Banga (8) that thermally contracted collagen is attacked more rapidly by elastase than is elastin itself. This observation leads to the suggestion that collagen fibrils are surrounded by a mucopolysaccharide envelope which is removed by heat denaturation and that collagen has the same structural elements as does elastin.

Ansell & Richter (5) confirm the existence of two previously reported peptidases and one protease in brain tissue and present evidence for an additional protease in that tissue. The distribution of dipeptidase activity in brain has been studied by Pope & Hess (108).

Levinson & Sevag (86) find the stimulating effect of manganese on the germination of *Bacillus megatherium* spores to be attributable to the activation of a protease by manganese. A purified protease has been isolated from cultures of *Streptomyces proteolyticus* by Tytell and co-workers (145). This enzyme appears to be zinc-activated and to have a specificity toward synthetic substrates similar to that of pepsin.

Carboxypeptidase.—Many of the successful applications of carboxypeptidase to the C-sequential analysis of proteins have been cited in other sections of this review. From studies of the rate of liberation of amino acids from corticotropin A, White (155) concludes that the C-terminal sequence is

Leu.Glu.Phe. Buchness & Friedberg (20) have studied the release of amino acids from ferritin by this enzyme, and Harris *et al.* (61) have studied the action of carboxypeptidase on hypophyseal growth hormone. Locker (89) has deduced C-terminal sequences for tropomyosin and for actin from the action of carboxypeptidase on these proteins.

Methods

Crook & Rabin (26) have used the observation that dipeptides form colored complexes with cobalt as the basis for a new assay for dipeptidase activity. Amino acids do not give a color but do cause deviations from Beer's law, presumably as a result of formation of mixed cobalt complexes.

Gorini & Lanzavecchia (50) have developed a modification of the milk-clotting assay involving the use of powdered milk suspended in buffer solutions. Ravin, Bernstein & Seligman (110) propose a method for the assay of chymotrypsin in tissue suspensions. The hydrolysis of the α-naphthol ester of acetylphenylalanine is followed colorimetrically after coupling the α-naphthol with tetrazotized diorthoanisidine.

Synthetic Reactions

Durell & Fruton (34) have made a straightforward analysis of the rather involved kinetic problem presented by the competitive transamidation which accompanies the papain or trypsin-catalyzed hydrolysis of benzoylargininamide in the presence of hydroxylamine. The reaction was formulated as:

$$\begin{array}{l} \xrightarrow[k_3]{H_2O} E + BA + NH_4^+ \\ E + BAA \underset{k_2}{\overset{k_1}{\rightleftarrows}} E.BAA \xrightarrow[k_4]{NH_2OH} E + BAH + NH_4^+ \\ E + BAH \underset{k_6}{\overset{k_5}{\rightleftarrows}} E.BAH \xrightarrow[k_7]{H_2O} E + BA + NH_2OH \end{array}$$

At 37° C., at pH 7.4, the value of the ratio k_5/k_1 for the papain-catalyzed reaction is 0.63 and the value of k_4/k_3 is 7.5×10^{-3}. It is calculated from the latter value that hydroxylamine is 420 times as effective as water in reacting with the enzyme-substrate complex. From the variation of k_4/k_3 with temperature, it was found that the energy of activation for hydrolysis is approximately 4.5 kcal. greater than the energy of activation for transamidation. The effect of pH on the system is consistent with the assumption that protonated hydroxylamine does not participate in transamidation. For the trypsin-catalyzed reaction k_5/k_1 is 9.4 and k_4/k_3 is 0.52×10^{-3}. It is apparent that trypsin is much more effective in catalyzing hydrolysis, as opposed to transamidation, than is papain. The question is raised whether this difference may be a general property of digestive enzymes as opposed to intracellular proteases.

Waley & Watson (151) have studied, by chromatographic methods, the intermediates formed during the hydrolysis of a variety of synthetic substrates by trypsin. In the case of dilysine ethyl ester the first reaction product is dilysine. Subsequently large amounts of tetralysine are formed and, at low trypsin concentrations, this product is very slowly hydrolyzed. The effect of pH on this system is consistent with the view that only uncharged amino groups are acceptors in transpeptidation reactions. Since a wide range of reactivity is covered by the substrates used and since transpeptidation is observed in every case, it is concluded that transpeptidation is not restricted to substrates of high activity. An accompanying paper by Blau & Waley (16) describes the intermediate reactions observed during the chymotryptic hydrolysis of amides, dipeptides, and dipeptide amides of phenylalanine and tyrosine. In general there is a less striking accumulation of products than was found with trypsin. Papain and ficin act upon tyrosinamide in essentially the same way as does chymotrypsin to yield tyrosine and dityrosine.

Fox, Winitz & Pettinga (43) have made a systematic study of the effects of various amino acid residues in the papain-catalyzed reactions between benzoyl amino acids and amino acid anilides. Benzoyl glycine undergoes synthetic reactions with the anilides of glycine, alanine, leucine, and valine to yield products of the type: bz.Gly.Gly.anilide. In contrast, benzoyl alanine participates in synthetic reactions only with the anilides of leucine and valine and undergoes transacylations with those of glycine and alanine. Further, the benzoyl derivatives of several amino acids undergo only transacylations with glycine anilide to yield the corresponding benzoyl amino acid anilides while the benzoyl derivatives of other amino acids do not react at all with glycine anilide. Since several synthetic reactions which are not found in this system have been observed with other enzymes or with other starting materials, the possibility that the products are not sufficiently insoluble to precipitate is ruled out. The authors infer that "it is the joint action of enzyme and substrate that contributes to the specificity of the reaction. This joint action may lead to a self-regulated order of residues." The term "zymosequential specificity" is coined for this concept.

Theory and Kinetics

Since, in many cases, at least one of the products formed by the action of proteases on synthetic substrates is a competitive inhibitor to some degree, kinetic constants have usually been evaluated from estimated initial reaction velocities. Foster & Niemann (42) point out that by the use of a Walker & Schmidt plot (152) all of the kinetic constants, including that describing product inhibition, can be evaluated.

In view of the concept that substrates are bound at more than one locus on an enzyme surface and that competitive inhibitors may be bound at an equal or smaller number of the same sites, Foster & Niemann (41) and Levine (85) have considered the kinetic problem which arises when each locus is

considered separately. Aside from the frequent examples of inhibition by high concentrations of substrate, a situation which was treated by Lineweaver & Burk (88), there appears to be little data at present which permits the effects of separate loci to be studied. This is probably the result of the high local concentration of a substrate or inhibitor with respect to other points of attachment when binding occurs at a single point.

A rather similar kinetic problem was investigated by Hagen & Jackson (57) who consider the possible equilibria arising between a metal-activated enzyme, assumed to have two anionic binding sites, and its divalent activator, and between the enzyme and the hydrolysis products of the metal ion. An expression is derived which, by the use of known hydrolysis constants, gives calculated curves in good agreement with the published data for the activation and inhibition of glycylglycine dipeptidase by cobalt and for the interaction between prolidase and manganese.

Klotz & Loh Ming (84) object to the view that the metal in metal-activated enzymes acts by chelating the substrate. The grounds for objection are that manganese and magnesium, the most common activating metals, are weak chelating agents and that the products of peptide hydrolysis are usually chelated more strongly than are the peptides themselves. It is proposed that the role of the metal is to stabilize the activated complex formed by the addition of hydroxyl ion to the peptide bond and that the metal, by virtue of its charge, also acts to increase the local concentration of hydroxide ion. Enzymatic specificity is assigned to the configuration of the enzyme surface. It is further suggested that, in the case of hydrolytic enzymes which do not require activating metals, the cationic side chains of arginine and lysine may serve the same surpose of stabilizing the activated complex.

A detailed theory of the action of carboxypeptidase and chymotrypsin is proposed by Ronwin (112). The primary postulate is that the oxygen and nitrogen of the susceptible peptide bond are bound to positive areas on the enzyme in such a way that electrons are pulled away from the susceptible bond. The bond then ruptures by mutual repulsion or by electron rearrangement followed by ionic attack. The postulate of homolytic fission, in the absence of experimental evidence for the existence of free radicals as intermediates, seems unnecessary. If the alternate route of decomposition of the postulated dipolar bond is accepted, the distinction between this theory and the familiar idea of bond polarization, recently given new impetus by the work of Swain & Brown (135), seems to be small. The theory also accounts, in a unified way, for the attachment of substrates to enzymes. While consistent arguments are made in the sense of electronic mechanism, steric effects were occasionally neglected so that the postulated points of attachment seem to be rather flexible.

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CHEMISTRY OF THE CARBOHYDRATES[1]

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Interest in the chemistry of the carbohydrates has continued to expand, and a large number of publications on sugars and related topics, have appeared during the past year. Certain aspects of carbohydrate chemistry, as for example the pathway of carbon dioxide in photosynthesis, are described elsewhere (1). Other important aspects must be omitted because of lack of space. The last review (2) dealt in the main with the chemistry of polysaccharides. This review will concentrate more on the chemistry of the monosaccharides.

The Enzymatic Synthesis of Monosaccharides

Certain details of this subject are treated elsewhere (1). Optically active sugars were prepared from smaller fragments many years ago (3); both chemical and enzymatic methods were used to separate the isomers. Meyerhof, Lohmann & Schuster (4) showed that muscle aldolase possesses the property of catalyzing the reversible aldol-type reaction between dihydroxyacetone phosphate and D-glyceraldehyde with the formation of a fructose derivative and that in the presence of another aldehyde a new sugar is formed. Thus it was demonstrated that the addition of L-glyceraldehyde and acetaldehyde lead to the formation of derivatives of L-sorbose and 5-desoxyketopentose respectively. It was stated that addition of propionaldehyde and formaldehyde also gave new unidentified sugars. An impure aldolase preparation from the pea has been shown to behave like muscle aldolase in that it will split hexose diphosphate (5) in the presence of other aldehydes with the production of new sugars. The addition of glycolaldehyde yields D-xylulose (6), while the tetroses D-erythrose and D-threose yield D-sedoheptulose and D-idoheptulose respectively (7, 8). All these substances are derivatives of D-xylulose and possess a *trans* configuration of hydroxyl group on C_3 and C_4. The presence of a phosphate grouping on the second aldehyde is not an essential for enzymatic synthesis to occur. The formation of 6-desoxy-L-sorbose, 6-desoxy-D-fructose (9), 5, 6-didesoxy-D-fructose, and 5-desoxy-D-ketoxylose (10) from DL-lactaldehyde, propionaldehyde, and acetaldehyde is in accord with this view. This indicates that the enzyme responsible for these transformations is possibly not aldolase but a 1-phosphofructo-aldolase (cf. 11), the fructose-1-phosphate perhaps arising from hexose diphosphate by enzymatic dephosphorylation (cf. 11a). The addition of formaldehyde-C^{14} to a mixture of dihydroxyacetone phosphate and liver extract yields L-erythrulose-1-phosphate (12). The reaction is reversible. Calvin *et al.* (13) have shown that sedoheptulose phosphate is an intermediate in photosynthesis,

[1] The survey of the literature pertaining to this review was completed in October, 1954.

and its formation from a tetrose and triose fragment (see above) indicates one way in which this sugar might be produced in photosynthesis. D-Xylulose is converted to D-xylose by an enzyme present in *Pseudomonas hydrophila* (14); no D-lyxose was detected. Thus it is possible that D-xylose arises from D-fructose via the reaction described above. The interconversion of D-glucose to D-galactose is now well substantiated (15, 16). Perhaps the enzyme responsible for this interconversion is capable of transforming D-xylose to L-arabinose (16a), 6-desoxy-D-fructose to D-fucose, and D-glucosamine to D-galactosamine by a similar mechanism.

Earlier it has been suggested that D-glucuronic acid and L-ascorbic acid might arise from the combination of two or three carbon fragments (17). The work of Isherwood *et al.* (18, 19) has demonstrated conclusively that in the cress seedling and rat these two carbohydrates are not formed by such a union. It has been shown that cress seedlings and rats will transform L-gulono-, L-galactono-, and D-glucuronofurano lactones and D-galacturonic acid methyl ester (cf. 20) into L-ascorbic acid. Cress seedlings convert D-altrofuranolactone and rats covert D-mannofuranolactone to D-arabo-ascorbic acid without inversion of the molecule. This conversion of derivatives of either D- or L-galactose to L-ascorbic acid suggests that D- and L-galactose and D-glucuronic and D-galacturonic acids are interconvertible in plants and that L-galactose and L-fucose may have a common origin. The metabolism of D-glyceric acid is in some way connected with the formation of Vitamin C from these lactones (21). The conversion of 6-phospho-D-gluconic acid to D-ribulose-5-phosphate is now well substantiated (22, 23). Perhaps D-glucuronic acid, which is a precursor of L-ketoxylose (24) (the ketopentose found in the urine of patients with pentosuria) is converted first to an L-gulonic acid derivative and thence by oxidation and decarboxylation to L-ketoxylose. If this should be so it would appear that these patients can take the metabolism of D-glucose one step closer to ascorbic acid than can the average human.

Branched Chain Sugars

Until recently only two branched chain sugars had been encountered in natural products, viz., apiose in apiin (25), and hamamelose in hamamelitannin (26). The branched chain sugar isosaccharinose was prepared from isosaccharinic acid many years ago (27) and dendroketose, which is related structurally both to apiose and hamamelose, was prepared from dihydroxyacetone in 1949 (28). New branched chain sugars have now been prepared from D-fructose via the cyanohydrin synthesis followed by oxidation of the resultant furano-lactone between C-5 and C-6 by metaperiodate. The product, $CO_2HC(CH_2OH)OHCHOHCHOHCHO$-(I), was reduced with hydrogen and the resultant hexonic acid converted by reduction with sodium amalgam to a reducing sugar, which was identified as 2-C-hydroxymethyl-D-xylose, an isomer of hamamelose. 4,4-Di-(C-hydroxymethyl)-D-threose, an analogue of apiose (29) was prepared from (I) by converting it first to the

ester-glycoside, followed by reduction of the ester grouping with sodium borohydride.

Other branched chain sugars have been found in the metabolic products of the fungi and bacteria (cf. 30). Streptose isolated from streptomycin was the first of these sugars to be investigated (31) and since then hydroxystreptose (32), cordycepose (33), cladinose, mycarose (34), tyvelose (35), abequose (36), the sugar in picromycin (37), and an isomer from rhodomycin (38) have been isolated and examined. The spatial configuration of the groups in streptose is known (30), but it has not yet been synthesized. The structure of cordycepose has been elucidated (39), and its DL-isomer has been prepared by the following elegant procedure (40). Bromoacetal was condensed with malonic ester, and the carboethoxy groups of the malonic ester derivative were reduced with lithium aluminium hydride to primary alcohol groups which were then acetylated. The product, $(AcOCH_2)_2CHCH_2CH(OEt)_2$, was heated with sodium hydrogen sulphate with the resultant loss of ethanol and formation of an unsaturated derivative $(AcOCH_2)_2CH_2CH=CH(OEt)$ which when oxidized with perbenzoic acid gave the epoxy compound from which DL-cordycepose was produced on hydrolysis. The *p*-nitrophenylosazone of the synthetic material contains no optically active centre and was identical with that of a specimen prepared from the natural product. Cordycepose is related to D-glyceraldehyde and streptose to L-glyceraldehyde; the configuration of the remaining branched chain sugars in unknown. Most of these sugars possess C-methyl residues, tertiary alcohol, and $—CH_2—$ groups in the molecule and show other points of resemblance. Another branched chain sugar, 3-desoxy-3-C-ethyl-D-altrose, has been prepared from the reaction of magnesium diethyl with 2,3-anhydro-4, 6-O-benzylidene-α-D-alloside (41). The reaction between potassium cyanide and 5-O-tosyl-1, 3-benzylidene-L-arabitol has led to the formation of 2-desoxy-L-galactonic acid, probably via a 4,5-anhydro derivative (42). It might be possible to use this type of reaction to synthesize branched chain sugars. Attempts have been made to forecast the configuration of the groupings, resulting from the opening of epoxide rings (43, 44). 1,3-Epoxide ring formation between secondary alcohol groups which has been encountered in cyclohexane derivatives has not yet been proved to occur in the sugar series (cf., however, 45). An interesting and unusual sugar, corchsularose, identified as a 2-desoxy-3-O-methyl pentose has been isolated from jute seed (46). Its structure requires further investigation.

Amino sugars are not commonly encountered in plant materials. However, D-glucosamine is found as chitin in some fungus polysaccharides and N-methyl-L-glucosamine has been isolated from streptomycin. The structure of the amino pentose present in the antibiotic puromycin has now been elucidated (47). This sugar was provisionally identified as a 3- or 4-aminopentose, since it reduced Fehling's solution, gave a negative ninhydrin test, and, after deamination, a positive furfuraldehyde test indicating the presence of a chain of five carbon atoms. Its N-acetyl derivative consumed only

two moles of periodate while the sugar reduced 3.8 moles. The following synthesis (47), which possesses several points of interest, has identified the sugar as 3-desoxy-3-amino-D-ribose. L-Arabinose was converted via the 2-O-toluene-*p*-sulphonate of methyl-β-L-arabopyranoside to methyl-2,3-anhydro-β-L-riboside, inversion occurring on C-2. When this substance was treated with ammonia methyl-3-amino-3-desoxy-β-L-xyloside was produced [cf. Hough & Jones (48)], identified as such because of the failure of metaperiodate to oxidize the derived N-acetyl derivative, which does not possess adjacent hydroxyl groups. The N-acetyl derivative yielded a 2,3-di-O-methanesulphonate which, when treated with sodium acetate in boiling alcohol was converted to the 2-O-acetyl-4-O-methanesulphonyl-3-N-acetyl-3-desoxy-methyl-L-lyxoside by inversion at C-2 probably via an oxazoline. The second methanesulphonyl group was displaced by sodium acetate in boiling 2-methoxyethanol solution; its replacement was also accompanied by Walden inversion, this time at C-4 and probably again via an oxazoline derivative to yield a derivative of 3-desoxy-3-amino-D-ribose, the sugar present in puromycin. The authors of this important paper comment that this synthesis passed through each of the four possible pentose configurations at various stages of these experiments. By a similar series of reactions 3-desoxy-3-amino-D-altrose derivatives were converted to the 3-desoxy-3-amino-D-allose series. The authors have substantiated their claim that "This use of neighbouring group inversions should be useful for the synthesis of some difficultly available sugars." They have used this effect to attain the total synthesis of the antibiotic, puromycin (49, 49a). In this case the starting material, a mixture of α- and β-methyl-D-xylofuranoside, was converted to the 3,5-O-isopropylidene derivatives which were separated into the isomers, each of which was converted to the ribose derivative in the following manner. On reaction with methanesulphonyl chloride the 2-O-mesyl derivative was produced which on hydrolysis with dilute acetic acid gave methyl-2-O-mesyl-α- (or β-) D-lyxofuranoside. When this substance was heated with sodium methoxide the mesyl group was eliminated and methyl-2,3-anhydro-(α- or β-) D-lyxofuranoside produced, treatment of which with ammonia at 100° C. opened the ethylene oxide ring with Walden inversion and with the formation of 3-amino-3-desoxy methyl-(α- or β-) D-arabofuranoside, characterized as the crystalline N-isopropylidene derivative. The amino grouping was first protected by acetylation and the methyl-3-acetamino-3-desoxy-D-arabofuranoside converted to the 2,5-dimethanesulphonyl derivative which when boiled with sodium acetate in methyl cellosolve was converted to methyl-2,5-di-O-acetyl-3-acetamino-3-desoxy-α- (or β-) D-riboside, which was then converted to 1-O-acetyl-2,5-di-O-benzoyl-3-acetamino-3-desoxy-D-ribose. This product was then condensed with the purine derivative from which puromycin was obtained. It is of interest that the same sequence of nitrogen, carbon, and oxygen atoms, NH_2—C—C(OH)—C—N—C—N, occurs in puromycin (starting at the sugar NH_2) as occurs in the guanido portion of streptomycin and that this sequence is that in a 5-hydroxy-6-amino pyrimidine.

The only other aminopentose of known configuration so far synthesized is 2-desoxy-2-amino-D-xylose which was prepared from D-glucosamine (50). Desosamine, a 3-desoxy-3-dimethylamino 4,6-didesoxyhexose ($C_8H_{17}NO_3$) has been encountered amongst the products of hydrolysis of the antibiotic erythromycin. The configuration of the groups within the molecule is uncertain. On oxidation with metaperiodate it yields a crystalline 2,3,5-tridesoxy-2-dimethylamino-pentose derivative (51). The sugar from picromycin is probably identical with desosamine. Both are readily converted by deamination and oxidation to crotonaldehyde (38). Carbomycin contains a branched chain dimethylamino sugar, $C_8H_{17}NO_4$ (52). The orgin of these unusual branched chain and amino sugars and the reason for their incorporation into the antibiotic molecule is unknown, but it is of interest that portions of the structures of chlortetracycline (Aureomycin) and oxytetracycline (Terramycin) show some points of resemblance to these sugars.

Relatively little is known of the origin of the two vitally important amino sugars D-glucosamine and D-galactosamine. However, experiments using labelled glucose indicate that D-glucosamine may be produced from this sugar without intermediate breakdown of the hexose molecule (53). It is unlikely that it is formed from tetrose plus aminoacetaldehyde (17), although the work of Metzler, Longenecker & Snell (54) suggests a mechanism whereby a tetrose might combine with glycine, a metal pyridoxamine derivative acting as intermediate, with the subsequent formation of glucosaminic acid. Leloir & Cardini (55) have shown that D-glucosamine is formed in the enzyme catalysed reaction between D-glucose-6-phosphate and glutamine. Heyns & Meinecke (56, cf. 57) have demonstrated that D-fructose and aqueous ammonia yield D-glucosamine. This evidence suggests that fructose-6-phosphate may be an intermediate in the scheme proposed by Leloir & Cardini.

The Degradation of Sugars by Alkalies

The action of ammonia and of lime water on reducing aldose sugars has been reinvestigated (58 to 62). The over-all effect is to cause isomerization of the aldose to a ketose which then either splits into smaller fragments or undergoes rearrangement to a saccharinic acid. When these fragments combine with ammonia heterocyclic rings such as glyoxalines are produced; pyrazine derivatives also may be formed. An interesting series of compounds possessing unusual properties are formed when secondary and primary amines are substituted for ammonia in this reaction (57, 63, 63a). Disaccharides are first isomerized to the ketose with alkalies and then split into fragments, one of which is the nonterminal sugar portion of the disaccharide. Kenner & Richards (64) have shown that the type of saccharinic acid [for separation of the saccharinic acid see (62, 65)] resulting from the action of oxygen-free lime water on hexose derivatives is determined by the presence or absence of O-alkyl or of O-glycosyl groups on C-3 or C-4. A substituent on C-3 hinders the formation of anions of the type $HOCH_2—CC(OH)=C—O^-$ and thus inhibits the formation of the D-glucosaccharinic acids and facilitates the forma-

tion of metaglucosaccharinic acids $CH_2OHCHOHCHOHCH_2CHOHCO_2H$, useful intermediates for the synthesis of the important sugar 2-desoxy-D-ribose (66, 67), which has also been prepared from D-glyceraldehyde by use of the Grignard reagent (68) or by means of the Reformatski reaction (69) and from 3-desoxy-D-glucose (70), 3-mesyl-D-glucose (70a) and D-arabinose (71). A substituent on C-4, as in the case of lactose, leads to the isolation of gluco- and isoglucosaccharinic acid $CH_2OHCHOHCH_2C(OHCH_2OH)O_2H$. These are branched chain sugar acids which are potential sources of some of the branched chain desoxy-sugars which occur naturally. Fully methylated sugars are much less susceptible to degradation by alkalies. 2,3,4,6-tetra-O-methyl-D-glucose, for example, will form sodium, potassium, lithium, and caesium compounds which can be used for disaccharide synthesis (72).

The separation of sugars and of their derivatives by chromatography and ionophoresis has become commonplace. A simple cheap ionophoresis apparatus has been described (73). Chromatography has been used to separate polysaccharides (74), and carbohydrases (75). Modifications in procedure and in the use of sprays are frequently encountered in the literature (76 to 82), and a method has been delineated which will differentiate between polyols and reducing sugars (83). Partition chromatography and chromatography on Florex XXX (84) have been used to separate the anomeric pyranose and furanose glycosides of D-arabinose (85) as well as those of D-ribose (86) and L-rhamnose. The synthesis of glucofuranosides from glucofuranolactone has also been described (87). Paper chromatography has also been used to separate and identify the methylene derivatives of L-rhamnose which are obtained when this sugar is heated with acidified paraformaldehyde. At least six derivatives were isolated and were characterized respectively as 2,3- and 3,4-O-dimethyleneoxy-L-rhamnose, 2,3- and 3,5-O-methylene-L-rhamnose, 1,3- 3,5-di-O-methylene-L-rhamnose, and an isomeric substance of unknown structure (88). D-Glucose yields a 1,2-3,5-di-O-methylene and a 4,6-O-methylene derivative (89), and D-xylose gives a 1,2-3,5-di-O-methylene derivative under these conditions. It is noteworthy that β-trans hydroxyl groups are frequently engaged in ring formation and that seven membered rings occur in some of these derivatives (cf. 90, 91). A successful attempt has been made to forecast the structure of methylene derivatives of the sugar alcohols using the principles of conformational analysis (92, cf. 93). Mills predicted that the methylenation of arabitol would afford the 1,3-2,4-di-acetal (*cis* junction with an equatorial CH_2OH group) rather than the 2,4-3,5-isomer (*trans* junction axial CH_2OH). This forecast was proved correct independently by Richtmyer & Zissis (94, cf. 95) who showed that D-arabitol gave at least three methylene derivatives, one of which was proved to be 1,3-2,4-di-O-methylene-D-arabitol, the second was 2,4-O-methylene-D-arabitol, and the third was identified as a most unusual type of sugar alcohol derivative which was composed of two molecules of the di-O-methylene compound joined by a methylene-oxy bridge, bis (1,3-2,4-di-O-methylene-5-D-arabityloxy) methane. Iditol yields di- and tri-benzylidene derivatives of unknown structure (96).

The Inositols

The identification of mesoinositol as a member of the B group of vitamines, the detection of derivatives of mesoinositol in streptomycin, and the possibility that inositol derivatives are intermediates in the pathway from aliphatic compounds to aromatic substances (97) have stimulated interest in the chemistry of the cyclic alcohols. Within the last year several interesting papers have been published on their chemistry and the determination of their spatial configuration. The syntheses of conduritol-B, scylloquercitol, and DL-viboquercitol have been announced (98). These substances were prepared from mesoinositol by first replacing a hydroxyl group by bromine by use of acetyl bromide. The resulting bromo-compound was caused to lose the elements of acetyl bromide by boiling it with zinc and acetic acid, leading to the formation of a cyclohexenetetrol (conduritol) derivative. If the desoxybromoinositol was reduced with hydrogen a quercitol resulted. The desoxybromoinositol reacted with sodium hydroxide with the formation of an epoxy compound (probably, cf. 63a). Substances containing carbonyl groups were not formed. This result compares with the reactions of sugar, 1,2-*trans*-halohydrins with sodium hydroxide (contrast the action of sodium hydroxide on *cis*- or *trans*cyclohexane 1,2-bromohydrin when either a cyclohexanone may be formed or ring contraction to a cyclopentanealdehyde may occur). McCasland & Horswill (99) have described the formation of a new dihydroconduritol from cyclohexadiene-1,4 by use of the Prevost reaction. This substance is an isomer of betitol, a cyclohexane tetrol which had been isolated from beet sugar molasses (100). The preparation from inositol dibromohydrin of further isomeric cyclohexane tetrols has also been described by McCasland & Horswill (101). Anderson *et al.* (102) have described the synthesis of DL-bornesitol and sequoyitol from mesoinositol.

The structures of the isopropylidene derivatives of the inositols have been examined by Angyal & MacDonald (103) and Anderson *et al.* (104). The former workers have shown that the isopropylidene group will, in certain circumstances, span *trans* hydroxyl groups. Thus the formation of an isopropylidene derivative is not necessarily proof that the hydroxyl groups which are engaged in ring formation are *cis* to one another. The structure of di-O-isopropylidene (−) inositol was proved by its oxidation with lead tetraacetate to a dialdehyde, which on reduction and subsequent hydrolysis gave L-mannitol. Ballou & Fischer (105) used a similar series of reactions to determine the configuration of (+) inositol. The configuration of the majority of the polyhydroxy derivatives of cyclohexane is now clear.

Several mono-O-methyl derivatives of the inositols are known. Comollo & Kiang (106) have described a di-O-methyl ether, dambonitol, which occurs in the latex from which chewing gum is manufactured. From a study of its oxidation with metaperiodate its failure to form O-isopropylidene derivatives and its conversion to mesoinositol after demethylation with boiling hydriodic acid, it was identified as 2,5-di-O-methyl mesoinositol. MacDonald & Fischer (107) have used their sulfone degradation procedure to open the

cyclohexane ring of scyllomesoinosose with the formation of xylo-trihydroxyglutardialdehyde. They have also degraded D-fructose ethyl disulphone to D-erythrose and the ethyl disulphones of aldohexoses and aldopentoses to pentose and tetrose sugars respectively. The ring structures of the disulphones have been investigated by Hough & Taylor (108, cf. 108a).

The origin and function of the inositols are not clear. Several attempts, for the most part unsuccessful, have been made to synthesize the inositols from sugar intermediates (but see 107). Wolfrom & Usden (109) have prepared several acylic dialdoses and have attempted to cyclise them to inositol derivatives, without success. The hexaphosphate of mesoinositol is perhaps a source of phosphate groups in plant metabolism. The shape of the inositols is similar to that of the sugars with pyranose rings; perhaps hydroxylated derivatives of cyclopentane and cycloheptane, similar in shape to the furanose and septanose sugars and precursors of the cyclopentane and tropanone ring systems, occur in natural products. Mesoinositol is encountered in the lipides (110) and is believed to play an important part in the synthesis of polysaccharides by yeast (111). The inositols resemble the sugar alcohols in their reactions. A D-galactosidyl-inositol occurs in molasses, a 2-D-galactosidyl glycerol has been isolated from seaweed (112), and a D-fructosidyl-glycerol has been prepared by the action of invertase on an aqueous solution of glycerol and sucrose (113).

It has long been known that tetritols, pentitols, and hexitols are oxidized, according to Bertrand's rule (114), by *Acetobacter xylinum* and *A. suboxydans* to ketose sugars. This rule was extended by Hudson, Richtmyer and their collaborators to include the oxidation of the 6-desoxypentitols and the inositols (115, 116). A polyol dehydrogenase present in rat liver, plants, and microorganisms, has been shown to convert D-sorbitol to D-fructose, L-iditol to L-sorbose, allitol to D-allulose, ribitol to D-ribulose, xylitol to D-xylulose, L-glycero-D-galaheptitol to perseulose and β-D-sedoheptitol to sedoheptulose. The other hexitols and 6-desoxyhexitols and cyclitols are not attacked. The following conditions are required for oxidation of the alcohol to occur. The secondary alcohol group attacked must have the L-configuration, it must be on the carbon atom β- to a primary alcohol group, and it must have on the γ-carbon atom a secondary alcohol group also with L-configuration. It is proposed to call this enzyme L-iditol dehydrogenase (117). Thus it is possible using either L-iditol dehydrogenase or Bertrand's method to convert ribitol to either D- or L-ribulose, allitol to D- or L-allulose, and sorbitol to D-fructose or L-sorbose. A mechanism for the conversion of sugars from the D- to the L- series and vice versa becomes apparent. Dulcitol is oxidized by neither of these enzymes, but there is very probably a galactitol dehydrogenase specific for the stereochemical pattern of dulcitol present in some microorganisms (118, cf. 18, 19).

Oxidations with Metaperiodate and Lead Tetraacetate

Some uses of these two reagents are discussed elsewhere in this article. The quantitative determination of formaldehyde and formic acid resulting

from the oxidation of sugars with metaperiodate or lead tetraacetate is frequently used to elucidate the structure of a sugar. Low yields of formaldehyde are attributed in some cases to the condensation of the aldehyde with an intermediate of the type $ROCH(CHO)_2$. Anomalous results may also accrue when the periodate determination is carried out by the arsenite method (119). To overcome these complications the oxidation is performed in the presence of an excess of benzaldehyde which combines with the reactive intermediate leaving the formaldehyde free; the periodate is best estimated by the thiosulphate method (120). Lead tetraacetate (121) and the Warburg respirometer (122) can be used to determine very small amounts of formic acid and to ascertain the position of union between the sugars in a disaccharide. These latter methods require only minute amounts of material and promise to be most valuable techniques.

From the action of sodium metaperiodate on 3-O-mesyl-D-glucose (70a, 123) crystalline 2-O-mesyl-4-O-formyl-D-arabinose has been isolated, thus proving the theory that formyl esters may result as intermediates during the oxidation of sugars by periodate (124). The oxidation by sodium metaperiodate of methylated derivatives of fructose has been studied by Mitchell & Percival (125) who found that the yields of formaldehyde were usually low and the uptake of periodate abnormally high. This work followed the synthesis of several methyl ethers of D-fructose which were prepared via the isopropylidene- and toluene-*p*-sulphonate derivatives of the ketose. The synthesis of 2-O-methyl and 3-4-di-O-methyl-D-galacturonic acid (126), 2,4- (127) and 2,5-di-O-methyl-D-glucose (128), 2-O-methyl-α-D-glucopyranoside (129, 129a), 2-O-methyl (88) and 4-O-methyl-L-rhamnose, 6-desoxy-3-O-methyl-L-idose, 2,4-di-O-methyl-L-rhamnose (130, 131), and 4-O-methyl and 2,4-di-O-methyl-D-galactose (132) and its 6-desoxy derivative (131) have also been described.

The increasing interest in the chemistry of the mucopolysaccharides has necessitated the preparation of derivatives of D-glucosamine and D-galactosamine. The 4,6-di-O-methyl ethers of both aminosugars have been prepared as well as the 6-O-methyl ether of D-glucosamine and the 3- (133) and 4-O-methyl ether of D-galactosamine (132). Hydrolysis of hyaluronic acid has given a disaccharide composed of D-glucuronic acid and D-glucosamine residues. By means of a series of reactions involving the reduction of the glucuronic acid carboxyl groups to CH_2OH with sodium borohydride, a disaccharide derivative was obtained which was degraded to 2-O-β-D-glucopyranosyl-D-arabinose (cf. 134). This same disaccharide was also obtained by degradation of laminaribiose (3-O-β-D-glucopyranosyl-D-glucose), thus proving that the glycosidic union between the uronic acid and glucosamine is of the β-type and that the hydroxyl groups on C-1 and C-3 are involved in the union of the two residues (135, cf. 136). Hyaluronic acid is considered to be composed of alternating units of β-D-glucopyruronic acid linked to N-acetyl-D-glucosamine (in its pyranose form) through positions 1 and 3; i.e., it resembles laminarin, a glucosan built up of β-linked sugar residues which are joined in the main through the hydroxyl groups on C-1 and C-3.

The acidic component of hyaluronic acid has been identified as D-glucuronic acid (137), and the presence of sulfamic acid groups in heparin has been recognized (138).

N-Acetyl-4-O-galactosyl-glucosamine, a derivative of lactose, has been isolated from the blood group substance of meconium. On hydrolysis it gave D-galactose and D-glucosamine. Its osazone, the infrared examination of which is recommended for identification, was indistinguishable from that of lactosazone, thus proving its structure (139). A disaccharide with the same structure has been isolated from the hydrolysis products of pig mucin (140, cf. 141, 141a). The synthesis of 6-N-acetyl-β-D-glucosaminido-D-glucose and of N-acetyl-6-O-β-D-glucosaminido-D-galactose have been described (139). These disaccharides differ from lacto-N-biose I and lacto-N-biose II, disaccharides composed of D-galactose and N-acetyl-D-glucosamine residues which have been isolated from the hydrolysis products of the oligosaccharide fraction of human milk. This oligosaccharide, beside containing D-glucose, D-galactose, and N-acetyl-D-glucosamine also contains L-fucose; its structure is as yet unsettled. Graded hydrolysis yields lacto-N-tetraose which is composed of a lactose molecule linked to a galactose residue via N-acetylglucosamine. The fucose containing oligosaccharide results from the acid hydrolysis of the Bifidus factor, an essential growth factor in milk (142). Its structure is possibly related to that of the blood group polysaccharides.

The isolation and chemical examination of the human blood group substances which possess A, H, and Le^a specificity have been described by Morgan and his colleagues (143). Gibbons & Morgan have described the isolation and properties of substances of human origin possessing blood group B specificity (144). Very careful fractionation of material from ovarian cyst fluid gave two substances, designated B and B^1 both of which possess blood group B character. Both substances gave L-fucose, D-galactose, D-glucosamine, and D-galactosamine on hydrolysis as well as 11 amino acids. Qualitatively there is no difference in the sugar composition of the B, B^1, A, H, and Le^a substances. D-Glucosamine, D-galactose and (?) mannose have also been detected in connective tissue (145). The effect of acids, alkalies, and periodate oxidation on these carefully purified substances has been determined. It will be noted that the sugar L-fucose occurs in these preparations. It also occurs in trace amounts in wood (146), is more commonly encountered in gums (147), and is readily prepared from the seaweed polysaccharide fucoidin (148). Methylpentose sugars are readily separated from the other monosaccharides by virtue of the fact that they are more tenaciously retained on a carbon column (149). The lower the ratio of hydroxyl grouping to carbon in the sugar the more difficult it is to wash the sugar off the carbon with water. Thus it is possible to separate on such a column mixtures of mono-, di-, tri-, and tetra-O-methyl-D-glucose (150). The antigen of *Shigella* contains methylpentose residues which have been identified as the more common methylpentose, L-rhamnose. D-Glucosamine is also present (151). L-Rhamnose together with D-xylose, D-glucose, and D-glucuronic acid have been encountered

as components of the polysaccharide sulphate in *Ulva lactuca* (152). A useful procedure for the micro-determination of acid, ester, and lactone links in polysaccharides, which depends upon the formation of hydroxamic acid derivatives and their subsequent colorimetric determination, has been described (153).

The Hydrolysis and Synthesis of Polysaccharides

The component sugars of polysaccharides are usually identified after hydrolysing them with dilute acids or with enzymes. If breakdown is not carried to the monosaccharide stage oligosaccharides can be isolated and examined. By this procedure a disaccharide composed of 1,4-α-linked D-galactose residues (154) and a 2-O-(D-galacto-pyruronosyl)-L-rhamnose (155) have been isolated from okra mucilage. This aldobiuronic acid has also been isolated from a mucilage present in grape juice (156). The di- and trisaccharides from apple pectin (157, 158) have been prepared and have been shown to be composed of D-galacturonic residues in the pyranose form joined by α-linkages to other D-galacturonic acid residues. These di-, tri-, and tetrasaccharides were prepared from pectic acid by enzymatic hydrolysis. Purification of these sugar acids was achieved by crystallization of the calcium or strontium salts, by chromatography on charcoal, cellulose or synthetic ion-exchange resins, and by crystallisation of the brucine salts (cf. 159, 160, 161). The specificity of the enzymes responsible for hydrolysis of the oligosaccharides from pectic acid has been determined from a study of the rate of hydrolysis of their methyl ester-methyl-glycosides, and of α-D-galacturonosyl-L-galactonic acid (162, 163). Several new di- and trisaccharides have been isolated from gums, mucilages, and hemicelluloses. A disaccharide composed of an L-arabopyranose residue linked β-glycosidically through C-3 to a second L-arabinose residue has been found in the products of hydrolysis of ϵ-galactan, cherry gum, peach gum, lemon gum, and golden apple gum (164). Disaccharides composed of a D-xylopyranose residue joined glycosidically through the hydroxyl groups on C-5, C-3, or on C-2 of an L-arabinose residue have been isolated from cholla gum (164) from golden apple gum (165), and from hemicellulose-B from corn cob (166). 3-O-Methyl-L-rhamnose has been detected in the products of hydrolysis of Scots Pine sawdust (166a). 4-O-Methyl-D-glucuronic acid (167) is probably the most commonly encountered O-methyl sugar in natural products, and as yet no other O-methyl derivative of glucuronic acid has been detected in nature. It has been found combined through C-4 of an L-arabinose residue (168) or joined through C-2 (169, 170, 170a), or C-3 (171, 172) of a D-xylose residue, union through C-2 being the most common. Glucuronic acid is usually united to other sugars via the hydroxyl group on C-4, and the conversion of this acid to its 4-O-methyl ether may be nature's way of preventing the formation of further branch points via this acid and thus to inhibit the formation of polysaccharides of a highly ramified form with gum-like characteristics. A crystalline acidic trisaccharide has been isolated from hemicellulose-B (173) and maltotetraose

and maltopentaose from starch hydrolysates (174). The detection and isolation of these sugars were facilitated by the use of paper chromatography and adsorption chromatography. These techniques are very sensitive and will permit the isolation of minute amounts of sugars. Because of the possible formation of oligosaccharides by acid reversion or enzymatic resynthesis during this degradation of polysaccharides, the interpretation of the isolation of disaccharides or of oligosaccharides should be treated with caution (175). For example on heating D-glucose in aqueous acid solution it is converted into oligosaccharides (176), into D-fructose (177), and into ω-hydroxymethylfurfuraldehyde. Thus the detection of gentiobiose or of a positive Seliwanoff test for fructose after hydrolysis of a glucose containing polysaccharide (cf. 178) is not sufficient proof that the disaccharide or ketose is a component of a glucosan. Carefully controlled blank experiments must be made to avoid such confusion (179). An added complication is that acids will convert some sugars to anhydro-derivatives. For example on heating sedoheptulose with acid sedoheptulosan, a substance provisionally identified as 2,7-anhydro-β-D-altroheptulofuranoside and 5-(1,2-dihydroxyethyl)-2-furfuraldehyde are produced (180). Hexose, heptoses, and heptuloses possessing the allo-, altro-, gulo-, or ido-configurations are prone to form anhydro derivatives of these types when heated with acids (181). Fructose and sorbose yield nonreducing dimers (182).

Polysaccharide Structure

Within recent years the emphasis has shifted from the purely exploratory work on the fine structure of the polysaccharides to the more dynamic approach of the biological origin of these polymers. The mode of formation of starch, glycogen, the fructans, and dextrans is now much clearer but is still far from completely understood. The origin of cellulose is less clear while evidence of the origin and methods of formation of the hemicelluloses, gums, and mucilages is completely lacking. The work of Peat and his colleagues has shown how α-D-glucose-1-phosphate can be converted to amylose and thence by the action of various enzymes, to amylopectin. One of these enzymes (Z) is a β-glucosidase which is capable of hydrolysing laminarin, cellobiose, and gentiobiose (183). This identification of the Z enzyme has been disputed by Hopkins & Bird (184) who consider it to be a weak α-amylase and defended by Peat & Whelan (185). Proof of the presence of multiple branching in waxy maize starch has been provided by Peat *et al.* (186) who treated the limit dextrin (from the action of β-amylase) with the debranching R enzyme (187) and examined the fractions of low molecular weight. This portion was shown to contain maltose, maltotriose, maltohexaose, and dextrins. Glucose, maltotetraose, and maltopentaose were not detected. The inference is that the internal chains (as distinct from branchings) cannot be less than six glucose units in length (cf. 174). This fact in conjunction with other evidence indicates that multiple branching is an intrinsic part of the amylopectin molecule. Hirst & Manners (188) have re-examined the evidence described above

and have calculated that the ratio of side chains to main chains is approximately one to four, and that, therefore, only one unit chain in five contained more than one branch point. Thus, the molecular structure approximates more closely a cigar shape than a sphere. Amylose contains a small number of oxygen sensitive bonds which are rapidly destroyed when the polysaccharide is heated under neutral conditions in the presence of air. Amylopectin is insoluble in boiling water if oxygen is rigorously excluded. It is also insoluble in 0.5 *N* sodium hydroxide at 20°C. in the absence of oxygen. This permits the separation of amylose from amylopectin by a simple extraction procedure (189). These observations indicate that caution must be used in the interpretation of the physical constants and structure of any polysaccharide which is isolated by an alkaline extraction procedure. It is generally agreed that the linkages in amylopectin are of the 1,4 or 1,6-α-glucosidic type and that a glucose residue is not attached to more than three glucose units. If this were not so, a substituted glucose polymer would result which would not be attacked by metaperiodate and which would yield glucose, detectable after hydrolysis of the periodate-oxidized molecule. Some evidence for the presence in waxy maize starch of an anomalous linkage has been obtained by Hamilton (190) who oxidized the polysaccharide with metaperiodate and reduced the resulting polyaldehyde with sodium borohydride. Methylation of the product followed by acidic hydrolysis gave a mono-O-methyl-D-glucose tentatively identified as the 4-O-methyl ether. If this identification is substantiated the presence of a glucose residue linked through C-1, C-2, C-3, and C-6 will be indicated. Bell & Manners (191) have oxidized glycogen with metaperiodate and have shown that over 99 per cent of the interchain linkages in glycogens from various sources are of the 1,6-type. The different physical properties of amylopectin and glycogen may, therefore, be caused by a variation in interchain linkages as well as by variations in the degree of branching. The glycogen molecule is more spherical than is that of the amylopectin. None of these glycogens contains fructose (cf. 192; contrast 177).

Many fructans contain D-glucose as part of the molecule and are considered to be formed from sucrose [the synthesis of which has been described (cf. 193 to 196)] by transfer of a fructose residue from one molecule of sucrose to another. This transfer is catalyzed by enzymes such as invertase, purified samples of which have been prepared (cf. 197), and results in the formation of tri- and oligosaccharides of varied structure. Kestose was earlier isolated and identified (198), and now the isolation and identification of neokestose as O-β-D-fructofuranosyl (2→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside is reported (199). Oligosaccharides of a similar nature have been isolated by Bacon (200) and have been prepared by the action of a transfructosidase from *Aspergillus niger* on fructose (201) and are known to occur naturally, e.g., in the artichoke and tulip bulb (202).

The architecture of the fructans has continued to attract attention because of their economic importance as components of the grasses. Schlubach and his colleagues have determined the structures of several new fructans

(203) and have observed that their molecular size varies from 16 to 50 hexose residues (204). It is very probable that these fructans consist of mixtures of polymer homologues, the average molecular weight of which depends upon the previous history of the plant. Methods for the determination of water soluble sugars in fresh and ensiled grasses have been elaborated by Wylam (205), and the rise and fall in fructose content during growth of the carbohydrate material of *Lolium perenne* have been examined by Schlubach & Holzer (206). The structure of a frucan fraction from *L. perenne* has been examined and shown to consist of a chain of D-fructofuranose residues joined glycosidically through C-2 and C-6 and terminated by a sucrose residue (207). The levans formed by *Pseudomonas prunicola*, Wormald, and *Bacillus subtilis* BG2F have been reinvestigated by Bell & Dedonder and have been shown to possess highly branched structures (208).

The chemistry and biochemistry of dextrans have attracted attention because of its importance as a blood plasma extender. It is generally accepted that a fraction of molecular weight of about $75{,}000 \pm 25{,}000$ is the most suitable, and several attempts have been made to prepare such material from crude dextran either by acidic (209, 210, 211) or enzymatic (212) breakdown of the high molecular weight polymer. The dextran may contain 1,6-1,4- and 1,3-linkages (cf. 213) and is prepared from sucrose by enzyme systems which transfer glucose residues to other molecules of glucose. If C^{14}-labelled sucrose is used, a labelled dextran results. This substance has been used to show that "the precipitation produced by human beings in response to the injection of small amounts of dextran is indeed antibody to dextran" (214). The structures of certain dextrans have been investigated very thoroughly and have been shown to contain small amounts of 1,3 linkages both by the metaperiodate oxidation procedure (215, 216), by the methylation technique (217), and by infrared spectroscopy. The aldehydic polymers resulting from oxidation of the dextrans with metaperiodate were reduced (215, cf. 218) and the resulting hydrogenated polymer hydrolysed. Glucose (3 to 5 per cent) resulted from residues which were joined by 1,3 linkages. A kinetic study of the rate of hydrolysis of NRRL B-512 dextran has shown that the branch points in the molecule are mostly of the 1,3-glycosidic type and that the external branch length is mainly one unit long (218).

Other substrates and enzymes can be used to prepare oligosaccharides. For example enzymes from *A. niger* (strain 152) will convert cellobiose into other β-linked glucose oligosaccharides (219); the enzymes in *Aspergillus flavus* behave similarly (220). The mechanism of synthesis of polymers from maltose (221), Schardinger dextrans (222), and from lactose (223) have also been investigated.

The commonest of all polysaccharides is cellulose, and yet despite intensive research in both academic and industrial laboratories much remains to be discovered about this polymer (cf. Symposium on Cellulose Chemistry held at St. Andrews, Scotland in 1954). The origin of cellulose is still unsettled. Brown & Neish (224) fed D-glucose-1-C^{14} to wheat plants and found

50 per cent of the glucose present in the cellulose to be labelled at C-1. Sorbitol is not an intermediate between glucose and cellulose. Greathouse (225) observed that glucose-1-C^{14} when fed to the cotton plant appeared as glucose-1-C^{14} in the cellulose. *A. xylinum* will convert glucose-1-C^{14} to the extent of 82 per cent to cellulose containing glucose labelled at C-1, the remainder of the activity was present at carbon atoms 3 and 4 in the glucose molecule (226). The inference is that the polymer is not formed solely directly from D-glucose residues but that some of the sugar residues are broken down and then resynthesized before they appear in the cellulose molecule. If ethanol was added with the glucose-1-C^{14}, the yields of cellulose were higher but the polysaccharide contained a lower percentage of C^{14}. Kursanov & Vyskrebentseva (227) have also studied the synthesis of cellulose in cotton fibers and believe it to take place via monosaccharides in the fibre itself, not in the leaves. The structure of the cellulose synthesized by *Acetobacter acetigenum* has been investigated and shown to be indistinguishable from that of a normal cellulose. Glucose and its derivatives and mannose are utilised by the organism for the manuf cture of cellulose (228).

Nitrocellulose has been found to react with liquid ammonia with the formation of an amino-cellulose which is insoluble in water and which possesses unique properties. The product behaves like an aromatic amine and can be diazotised, and the diazonium compound coupled with aromatic substances. The diazotised product on reduction with hypophosphite yields a desoxycellulose which contains only 1 to 2 per cent of nitrogen. If di-N-butylamine is used instead of ammonia, nitrocellulose yields a di-N-butylamino-cellulose (229). Toluene-*p*-sulphonyl derivatives of cellulose have also been converted to aminocellulose (230).

Hemicelluloses

No article on carbohydrate chemistry would be complete without some mention of these polysaccharides. They abound in plants and are usually considered as waste material, to be disposed of in as economic a manner as possible. However, within recent years this group of polysaccharides has been shown to be of importance in bread baking and in paper manufacture, to quote but two examples. The structure of the hemicellulose fraction from barley has been investigated (231) and shown to be composed of at least three polysaccharides, a β-linked glucosan similar to cellulose, an α-linked glucosan similar to amylose, and a branched chain xylo-araban. The hemicellulose from birch sawdust has been identified as a xylan composed of 24 residues of D-xylose and one of 4-O-methyl-D-glucuronic acid (232). The xylan from wheat straw has been shown to be composed of D-xylopyranose residues, L-arabofuranose units, and aldobiuronic acids identified as a monomethyl derivative of 3-O-α-D-glucuronosyl-D-xylose and 6-O-β-D-glucuronosyl-D-galactose (234) which has also been detected in the products of hydrolysis of *Acacia pycnantha* (233). Cautious hydrolysis removed the arabinose residues and most of the uronic acid groups and left a chain of *ca.* 30 D-xylose

residues (234). Aspinall & Mahomed (235) found no L-arabinose or D-galactose in their preparation of wheat straw xylan. They concluded that the polysaccharide was devoid of arabinose residues and that it was composed of 40 to 45 D-xylopyranose residues and that a 3-O-α-D-glucuronosyl-D-xylose was part of the polysaccharide structure. Hemicellulose A from beech wood resembles wheat straw hemicellulose in that it is composed of a long chain of D-xylopyranose residues (*ca.* 70) but differs in that the D-glucuronic acid present is linked through C-2 of a xylose molecule and not through C-3 (236). There is some evidence that xylans may contain small amounts of L-rhamnose as a component. The main differences in the xylans may therefore be in the mode of linkage of the uronic acid residues and in the presence or absence of L-arabinose residues. The work of Aspinall, Hirst & Mahomed (236) suggests that xylan is free from L-arabinose residues and that any detected arise from the hydrolysis of araboxylans, arabogalactans, or arabans associated with and very difficult to remove from xylan. On the other hand the work of Roudier (234) suggests that L-arabinose is a component of the (straw) xylan molecule.

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CHEMISTRY OF THE PHOSPHATIDES[1,2]

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During the years covered by this review many significant advances in the chemistry of the phosphatides have been reported. These have been attributable in large measure either to new techniques or to refinements of existing ones. Among the more significant accomplishments are the isolation from baker's yeast of the first natural lecithin containing two unsaturated fatty acids, and of 13 individual lipopeptides from blood serum. The synthesis of sphingine and of *erythro* and of *threo*-dihydrosphingosine, which has yielded valuable information as to the structure and configuration of the natural compounds, was achieved. L-α-glycerylphosphorylethanolamine, L-α-distearoylphosphatidyl L-serine, and two glycollecithins, the latter being members of a new class of synthetic phosphatides, were synthesized. A chemical degradation procedure that permits the elucidation of the structure and configuration of naturally occurring cephalins and phosphatidyl serines by a simple and rapid method was discovered.

Separation of Phosphatides

Chromatographic methods.—Borgström (1) investigated the separation of phosphatides from neutral fat and free fatty acids on saccharose, magnesium oxide, or silicic acid columns, using glycerides and fatty acids labelled with C^{14} as tracers; the degree of separation with silicic acid is superior to that obtained either by saccharose or magnesium oxide, or by the precipitation method with acetone. Lea & Rhodes (2) studying the partition chromatography of egg-yolk lecithin on paper, or on cellulose columns found, in contrast to Bevan *et al.* (3), that no useful separation of the amino- and choline-containing phosphatides is achieved. Chromatography on cellulose affords, however, an excellent method for the removal of free amino acids accompanying phosphatides, or, as Smith reports (4) of the water-soluble impurities of latex lipides. The application of the method to crude egg-yolk phosphatide reduced the atomic ratio of N/P from 1.12 to 1.0. The separation of egg-yolk phosphatides, free of ether-insoluble material and contaminating amino acids (2), was achieved by Lea & Rhodes (5) by adsorbing the mixture on a column of activated silicic acid and celite, and eluting the phosphatides with suitable mixtures of methanol and chloroform. The whole of the amino-containing phosphatides was recovered free of choline in a fast running,

[1] The period reviewed covers the interval from January, 1952, to October, 1954. Two publications from the author's laboratory appearing in January, 1955, have been included.

[2] The following abbreviations are used in this chapter: GPC for glycerylphosphorylcholine; GPE for glycerylphosphorylethanolamine; PC for phosphorylcholine; PE for phosphorylethanolamine.

cleanly separating fraction. The choline-containing fraction that followed possessed the theoretical content of phosphorus and the correct ratios of N/P and fatty acid/P for lecithin. The final elution with methanol yielded a lysolecithin fraction.

Descending chromatography on paper was used by Huennekens, Hanahan & Uziel (6) for the separation and identification of lecithins and their degradation products. The chromatograms were run at room temperature (25°C.) on 50×100 cm. sheets of Whatman No. 1 paper in chromatographic cabinets. The following solvent systems were found to be most satisfactory: (*a*) *n*-butanol saturated with water, (*b*) ethanol-water (8:1), (*c*) *n*-propanol-water (8:1), and (*d*) *n*-propanol-acetic acid-water (8:1:1). In general, the principal use of the solvent systems is to separate or identify the following classes of compounds: (*a*) lecithins, (*b*) lysolecithins, (*c*) phosphatidic acids, and (*d*) derivatives of (*a*) in which the fatty acids have been removed. Furthermore, within a given class it is possible to resolve certain mixtures such as lecithin and lysolecithin, saturated and unsaturated lecithins or the corresponding lysolecithins, and mixtures of choline, PC[2], GPC[2], and glycerophosphate.

A chromatographic technique for the separation of inositol phosphates by means of ion exchange has been described by Smith & Clark (7). The method was applied successfully to the separation of sodium phytate and a mixture of organic phosphates obtained by the action of phytase on sodium phytate. By the use of the weak base anion exchanger, D-Acidite, as an adsorbent, and HCl (0.1 to 1.0 *N*) as an eluant, nine organic phosphorus compounds were obtained in sufficient quantities to permit the determination of their inositol to phosphorus ratios. Sodium phytate, which has been considered sufficiently pure for use as a standard, was shown to contain some 30 per cent of compounds other than the hexaphosphate. The pentaphosphate accounted for the bulk of impurities.

A separation of the water-soluble nitrogenous constituents of liver into two groups by means of the ion-exchange resin, Zeo-Karb 225, was accomplished by Campbell & Work (8). Among the group of compounds not adsorbed on Zeo-Karb were three ninhydrin-positive substances, which were identified as taurine, ethanolaminephosphoric acid, and GPE[2].

Countercurrent distribution methods.—The method of countercurrent fractionation has been applied by Cole, Lathe & Ruthven to the separation of lipide material from human brain (9). A solvent system (A) composed of 62 per cent chloroform, 35 per cent methanol, and 3.15 per cent water (v/v/v) was devised to minimize the formation of emulsions. By the use of solvent system A, crude brain lipide as well as preparations of lecithin, cephalin, and a mixture of sphingomyelin and cerebroside, prepared by the customary methods from crude brain lipide, were distributed through 49 tubes in the glass and polythene apparatus described by Lathe & Ruthven (10). The distribution of crude brain lipide in system A gave rise to three groups of fractions, a very polar one (tubes 0 to 4), a polar one (tubes 5 to 17), and one of

intermediate polarity (tubes 18 to 40). The analysis for N, P, sugars, and cholesterol, and the identification of bases by paper chromatography, showed the relative positions of a number of recognized lipides, although there was a considerable overlapping of many components. Amino acids, reducing material, and breakdown products were present in the most polar tubes (4 to 16). In the central region peaks were found as follows: sphingomyelin (tube 21), lecithin (tube 27), cerebroside (tube 29), and cholesterol (tube 35). A small amount of nonpolar material was present in the position of neutral fat (tube 45). For further separation of the polar fractions the solvent system A was altered by the addition of chloroform and methylene chloride, or, as an alternative, the phase ratio (upper to lower) was changed from 1/1 to 1/4. It appears that the nature of the phosphatide base is the main factor which determines its distribution between the solvent phases. In the cephalin group the degree of unsaturation and length of the fatty acid may be of equal importance. Countercurrent fractionation of lipide material from human placenta (11) gave fractions with nitrogen and phosphorus values indicative of the presence of atypical phosphatides with high phosphorus and low nitrogen content. Lovern (12) found that a useful degree of separation of lecithin from similar lipides, containing ethanolamine or serine could be effected by countercurrent distribution between light petroleum (b.p. 40–60°C.) and aqueous ethanol (85 per cent v/v). The separation of cephalin from phosphatidyl serine, however, is inadequate.

The effect of solute concentration on the countercurrent distribution of phosphatides was studied by Olley (13). Using the original all-metal twenty-tube countercurrent apparatus of Craig (14), Olley observed that the distribution of crude phosphatide mixture, obtained from an acetone extract of haddock muscle, between light petroleum ether and acetone-ethanol-water, gave widely varying distribution patterns, depending on the concentration of the phosphatide. A high concentration of the phosphatide favoured distribution into the petroleum phase, whereas with increasing dilution more of the extract tended to go into the acetone-ethanol-water phase. These observations were found to apply also to the total phosphatides of groundnut and soybean when distributed between light petroleum and 85 per cent ethanol. Of interest also, is Olley's (15) observation that phosphatidic acids readily undergo hydrolysis in 85 per cent ethanol and that the liberation of the fatty acids is accompanied by phosphoric acid migration. The progress of the hydrolysis, which apparently is not attributable to a pH effect, was followed by countercurrent distribution using light petroleum as the second phase.

Solvent separation.—Wik, Scholfield & Cowan (16), investigating the efficiency of isopropyl alcohol for the fractionation of soybean phosphatides, found that it has many advantages as a commercial solvent and gives a good separation of the inositol-containing fraction from the other phosphatides.

Identification of phosphatides by paper chromatography.—As Hack (17) has pointed out there is a need in lipide research for rapid and sensitive

methods for the separation and identification of the lipide components of mixtures obtained by the extraction of tissues. The author describes a chromatographic technique for the separation of lipides in quantities of 1×10^{-2} μM or less, on single filter paper disks, and for the identification of the components by spot tests. The filter paper chromatogram is cut into two or more sectors, and each piece is tested with a different reagent in order to characterize the sample fully. The small amount of tissue required (1 mg.) makes it possible to use the technique as an adjunct to histochemical studies of lipides. Caution, however, must be exercised because some of the color reactions described by Hack are given also by a variety of nonlipide nitrogenous materials which occur as constituents of proteins.

Paper chromatographic methods were also used by Hecht & Mink (18) in their investigation of the composition of the cephalin fraction involved in the clotting of blood. The partially purified cephalin preparations were chromatographed on Whatman No. 1 filter paper by the method of Consden, Gordon & Martin (19), using as solvent pairs phenol-water, phenol-ammonia, butyl alcohol-water, butyl alcohol-pyridine, and isobutyric acid-water; the chromatograms were developed with ninhydrin in butanol.

A rapid test for the differentiation of easily and difficultly hydrolyzable phosphoric acid esters on paper chromatograms has been described by Fleckenstein, Gerlach & Janke (20). The chromatograms are sprayed with a molybdate reagent and, while still wet, are heated to 85°C. for a period of 7 min. On exposure to hydrogen sulfide the phosphorus-containing spots develop a blue color which disappears in a few seconds in an atmosphere of ammonia if the color is caused by difficultly hydrolyzable esters (adenylic acid, inosinic acid, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, glycerolphosphate, phosphoglyceric acid, inositolhexaphosphoric acid) but remains if caused by inorganic phosphate or readily hydrolyzable phosphoric acid esters.

Analytical Methods

No essentially new methods for the quantitative determination of either phosphorus or the nitrogenous components of phosphatides have been reported in the period under review. Most methods reported are adaptations of older methods to micro quantities.

Phosphorus.—Ma & McKinley (21) described a colorimetric microprocedure for the determination of phosphorus in samples weighing from 3 to 10 mg. and containing from 6 to 22 per cent of phosphorus. The procedure is based on the yellow color of a soluble complex produced by mixing ammonium vanadate, molybdate, and phosphate and gives results of about the same degree of accuracy as the gravimetric method, but is faster. A precision of ± 0.2 per cent of phosphorus is obtained with samples weighing from 3 to 10 mg. Other colorimetric methods for the determination of phosphorus in amounts of 300 μg. and less have been described by Takahashi (22), Nakamura (23), and Soyenkoff (24).

A method for the volumetric determination of phosphorus has been reported by De Lorenzi & Aldrovandi (25). The inorganic phosphate, obtained by the digestion of the organic substance with a mixture of sulfuric acid and nitric acid, is precipitated with a measured volume of a magnesium chloride solution in the presence of ammonium salt and ammonia. The excess of magnesium in the filtrate is titrated with a 0.1 *N* solution of Complexon III, using as indicator a 0.4 per cent solution of Erichrome T Black in ethanol.

Choline.—A colorimetric method for the quantitative determination of choline, based on the deep yellow color of its 2,4,6-hexanitrodiphenylamine complex in acetone solution, has been reported by Samuelsson (26). Since other organic bases also form colored complexes with hexanitrodiphenylamine the method is not specific for choline. Its accuracy is claimed to be within 2 per cent of the theoretical values, and the results are in good agreement with those obtained by the reineckate method.

The observation of Appleton *et al.* (27) that choline periodide is soluble in ethylene dichloride and in this solvent exhibits an ultraviolet spectrum which differs markedly from that of free iodine, was developed into a method that permits the estimation of as little as 5 μg. of choline. Marquardt & Vogg (28) report the determination of choline with sodium tetraphenylboron. Potassium and ammonium ions interfere, but proteins and amino acids do not.

Glycerol.—A rapid, yet fairly accurate, determination of glycerol in aqueous solution by oxidation with potassium dimesoperiodate ($K_4I_2O_9$ or $K_2H_3IO_6$) has been developed by Hartman (29). The dimesoperiodate, with a solubility 20 times as great as potassium metaperiodate, makes it possible to increase the size of the glycerol sample and achieve greater reproducibility.

Nitrogenous bases.—A separation by chromatography and the quantitative estimation of the nitrogenous lipide constituents, ethanolamine, serine, and choline in quantities ranging from 5 to 75 μg. has been described by Levine & Chargaff (30). The method consists of the following steps: (*a*) hydrolysis of the phosphatide and removal of the fatty acids, (*b*) separation of the bases by one-dimensional partition chromatography on filter paper, (*c*) demonstration on chromatographic guide strips of the location of adsorption zones containing primary amines by means of ninhydrin, (*d*) quantitative colorimetry of the corresponding eluates, (*e*) development of separate chromatograms for choline by its conversion to choline phosphomolybdate followed by the reduction of the latter to molybdenum blue, and (*f*) quantitative planimetry of the choline spots.

Phosphatides.—A procedure for the direct determination of phosphatidyl serine and cephalin in red blood cells and in plasma has been described by Axelrod, Reichenthal & Brodie (31). The method involves the extraction of the phosphatides, their hydrolysis by alkali, and formation of colored derivatives of ethanolamine and serine with dinitrofluorobenzene. These derivatives are separated by their differential solubilities in organic solvents and then are assayed spectrophotometrically at 420 mμ. As a rapid and sensi-

tive method for the estimation of amino-N in intact phosphatides, the ninhydrin procedure has been recommended by Lea & Rhodes (32). If carefully purified reagents are used 1 to 5 μg. of N in cephalin can be determined in the presence of up to 1000 μg. of N occurring in nonamino phosphatides.

Thannhauser *et al.* (33) reported a chemical method for the quantitative determination of GPC and of the preformed choline fraction in tissues. The method makes use of the fact that GPC is quantitatively hydrolyzed to choline and glycerophosphoric acid within 20 min. by 1 *N* HCl at 100°C., whereas PC is hydrolyzed to a negligible extent under these conditions. Thus the difference between the amounts of choline reineckate obtained before and after hydrolysis represents the amount of choline originating from GPC, if other readily hydrolyzable choline compounds are absent. Interfering contaminants can be removed by mercuric acetate without loss of the choline compounds.

Naturally Occurring Phosphatides

Lecithins.—The isolation of the first natural lecithin to contain two unsaturated fatty acids was reported by Hannahan & Jayko (34). The isolation of this substance is of interest since a lecithin with two saturated fatty acids has already been isolated and thus the concept that natural lecithins invariably contain both a saturated and unsaturated fatty acid in each molecule has now been invalidated. The unsaturated lecithin was obtained from baker's yeast (*Saccharomyces cerevisiae*) and was identified as dipalmitoleyl glycerylphosphorylcholine. The unsaturated lecithin, a pasty, colorless substance ($[\alpha]_D+6.6°$, iodine number 67.2) was found to be quite stable to atmospheric oxidation. Its catalytic hydrogenation yielded a dipalmitoyl lecithin ($[\alpha]_D+6.6°$) which was identical with authentic L-α-(dipalmitoyl) lecithin ($[\alpha]_D+6.6°$) (35). The dipalmitoyl lecithin hence possessed the α-structure and the L-configuration. The same structure and configuration has to be assigned to the individual lecithins of the dextrorotatory lecithin mixture ($[\alpha]_D+5.36°$) obtained from human brain by Klenk *et al.* (36). The α-structure and L-configuration was also established for the phosphatidyl moiety of phosphatidyl serine from ox brain (37, 38). The proof of the L-α-structure for both the unsaturated yeast lecithin and ox brain phosphatidyl serine lends further support to the theory advanced by Baer and his associates that the natural glycerolphosphatides most likely occur only as α-isomers and have the L-configuration.

Lipopeptides.—In the course of their investigation of the lipopeptides of blood serum, Schrade and co-workers (39, 40), by means of a combination of one- and two-dimensional paper chromatography, succeeded in isolating 13 individual lipopeptides. The phosphatide-like nature of the lipopeptides, suggested by their solubility characteristics, was confirmed by positive reactions for choline and fatty acids, and the fact that the ninhydrin-positive spots given by lipide extracts of rats treated with P^{32} were radioactive. Acid hydrolysis of the lipopeptides and separation of the amino acid mixtures by

two dimensional paper chromatography revealed that the peptide chains contained from 3 to 11 different amino acids. The following amino acids were found to be present: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, leucine, isoleucine, methionine, norleucine, norvaline, hydroxyproline, phenylalanine, proline, serine, threonine, tyrosine, and valine. The isolation of phosphatidyl peptides from plasma and red blood cells of man and horse has also been reported by Macheboeuf *et al.* (41). Among the amino acids identified as constituents of the peptide chains were glutamic acid, serine, glycine, alanine, and arginine.

Barbier & Lederer (42) reported the isolation of a phosphatide from a strain of *Mycobacterium phlei* grown on Santon's medium. The phosphatide, on repeated reprecipitation from ether with acetone, was obtained in the form of a yellow powder (m.p. 180 to 190° C., P, 1.1 per cent; N, 0.62 per cent). The paper chromatogram of the intact phosphatide gave a single spot reacting with ninhydrin, and practically all of the phosphorus was contained in this spot. On acid hydrolysis it yielded an amino acid which by paper chromatography, electrophoresis, oxidation with periodic acid, and the formation of two picrates (m.p. 110°–115°C. and 195°C.) was identified as hydroxylysine. By analogy with phosphatidyl serine, Barbier & Lederer assume that the phosphatidyl group is attached to the hydroxyl group of hydroxylysine.

Polyglycerolphosphatide.—Using a combination of solvent fractionation and chromatographic procedures for the resolution of the alcohol-insoluble phosphatides of dog liver, McKibbin & Taylor (43) succeeded in isolating a phosphatide that was entirely free of inositol phosphatides and contained only minimum amounts of nitrogenous phosphatides. It contained glycerol, phosphorus, and fatty acids in the molar ratios of 3:2:3, and on acid hydrolysis yielded glycerophosphoric acid and a phosphate ester of glycerol in combination with an as yet unidentified substance. The possibility that the new substance, most likely a polyglycerol phosphatide, is an artefact has been considered by McKibbin & Taylor. The methods employed, however, seem to minimize this possibility.

Inositol phosphatides.—Faure & Morelec-Coulon (44), fractionating a mixture of phosphatidic acids obtained from wheat germ, succeeded in isolating a microcrystalline sodium salt of a phosphatidic acid. The substance on acid hydrolysis yielded a mixture of approximately equal amounts of palmitic acid and an unsaturated C_{18}-acid (average M = 263), glycerol, phosphoric acid, and inositol in the molar ratios of 2.1, 0.96, and 1.05 for fatty acids/phosphorus, glycerol/phosphorus, and glycerol/inositol, respectively. By analogy with other naturally-occurring phosphatides, the structure of an α-phosphatidyl inositol was tentatively assigned to the phosphatidic acid. The available evidence, however, was not sufficient to exclude other structures. Somewhat later the same investigators, in the fractionation of cardiolipin, (45) obtained a phosphatidyl inositol which differed from that of wheat germ in that it contained stearic acid in place of palmitic acid, and that its unsaturated fatty acid had an iodine number of 220, instead of 155.

In an extension of earlier work, but with chromatographic checks, Malkin & Poole (46) succeeded in obtaining a more homogeneous inositol-containing phosphatide fraction which comprised some 38 per cent of the phosphatide present in groundnuts, and contained ethanolamine as the only base. The substance, a colourless powder, darkened slightly on storage and gave the Scherrer test for inositol, the acraldehyde test for glycerol, and the Molisch test for carbohydrates. Each molecule of phosphatide contained two molecules of L-arabinose and one of D-galactose, which were combined through glycosidic linkages. The substance differed from other phosphatides, in having a nitrogen/phosphorus ratio of 1:2. Its degradation products seem to be consistent with the structures of an N-glycosyl derivative of the ethanolamine ester of phosphatidylinositol phosphate.

Hawthorne & Chargaff (47) separated chromatographically the water-soluble phosphates that were released by brief acid and alkaline hydrolysis from soy bean inositol phosphatides, and found that the hydrolysis products, in addition to inositol monophosphate, consisted of phosphoric acid esters which were thought to be the galactoside and arabinoside, respectively, of inositol monophosphate.

Cerebrosides.—A carbohydrate-containing phosphatide (globoside) was isolated from human blood stroma by Yamakawa & Suzuki (48). The globoside, which was obtained in colorless and fairly hydroscopic spherocrystals (sintering 200°C., m.p. 224°C.), on hydrolysis yielded lignoceric and nervonic acids, D-galactose, chondrosamine, and a sphingosine-like substance. Thus the globoside is most likely a mixture of lignoceryl- (or nervonyl-) sphingosine-digalactoside and of lignoceryl- (or nervonyl-) sphingosine-acetylchondrosamine-digalactoside. The isolation of a glycolipide consisting of 25 per cent of lignoceric acid, 16 per cent sphingosine, and 56 to 57 per cent of sugar (galactose, glucose, glucosamine) from the stroma of bovine red blood cells has been described by Klenk & Lauenstein (49).

Constituents of Phosphatides

Fatty acids.—Klenk, in a continuation of his investigation of the composition of the fatty acid components of brain phosphatides, undertook with Bongard (50) a study of the highly unsaturated C_{20} and C_{22} fatty acid fraction. Separation of the acids was carried out by fractional crystallization of their methyl esters at low temperature, followed by fractional distillation *in vacuo*. All fractions, on oxidation with ozone, gave malonic acid in yields of over 50 per cent of theory, indicating that the highly unsaturated fatty acids of brain matter consist almost exclusively of polyethanoic acids of the divinylmethane type. In addition, the C_{20}-acids yielded caproic acid, pelargonic acid, glutaric acid, and a C_{11}-dicarboxylic acid; and the C_{22}-acids gave propionic acid, caproic acid, pelargonic acid, succinic acid, and pimelic acid. Hence the following four C_{20}-acids: Δ^{11}, $\Delta^{11,14}$, $\Delta^{5,8,11}$, and $\Delta^{5,8,11,14}$; and six C_{22}-acids: $\Delta^{7,10,13}$, $\Delta^{4,7,10,13}$, $\Delta^{7,10,13,16}$, $\Delta^{4,7,10,13,16}$, $\Delta^{7,10,13,16,19}$ and $\Delta^{4,7,10,13,16,19}$ can be considered as possible components of the mixture of highly unsatu-

rated fatty acids. Definitely identified were the Δ^{11}-eicosenoic acid and arachidonic acid ($\Delta^{5,8,11,14}$-eicosatetraenoic acid). The presence of docosahexaenoic acid seems, however, practically certain.

In 1952 Niemann and associates (51) showed that the agent formed by *Escherichia coli* that produces a hemorrhagic response in experimental mouse sarcoma 180 and causes its regression, is a complex polysaccharide that contains both a peptide and a phosphatide component. When the hemorrhagic agent was subjected to a partial hydrolysis (52) with aqueous sulfuric acid, a chloroform-soluble, water-insoluble fraction was obtained that could be separated into fractions, soluble and insoluble in acetone. The latter fraction, apparently a phosphatide, on hydrolysis with hydrochloric acid gave, in addition to lauric, myristic, and palmitic acids, an optically active β-hydroxymyristic acid to which provisionally the D-configuration was assigned.

Under the usual conditions of crystallization from hot solvents the α-hydroxy-*n*-fatty acid constituent of yeast cerebrin separates as a gel. By taking the precaution of lowering the temperature of its solution in dry ethanol very slowly from 50° to 33°C., Chibnall *et al.* (53) obtained a crystalline material. The x-ray photographs of the crystals showed the characteristic intensity distribution of an α-hydroxy-*n*-fatty acid. The spacings indicated, however, that a small amount of homologous contaminant was present. Melting points and x-ray data for the *n*-fatty acids obtained by oxidation revealed that the cerebrin hydroxyacid is α-hydroxy-*n*-hexacosanoic acid with not more than 10 per cent of α-hydroxy-*n*-tetracosanoic acid. A reinvestigation of the structure of α-hydroxy nervonic acid by Klenk & Faillard (54) revealed that it is not, as was thought, pure Δ^{15}-*n*-α-hydroxy-tetracosenoic acid but a mixture of the Δ^{15}- and Δ^{17}-*n*-α-hydroxy-tetracosenoic acids.

The fatty acid composition of egg yolk lipides has been investigated by Shorland (55). Lea & Rhodes (56) claim that the method of Yasuda for determining iodine values, when applied to phosphatides, gives results that are much too low.

Aldehydes.—The plasmal content of the glycerolphosphatides of heart, pancreas, kidney, adrenals, liver, intestines, ovary, and spleen was determined both by a modified Feulgen method and by isolation of the aldehydes in the form of their dimethyl acetals. In most cases the values obtained by the two methods agreed satisfactorily (57). On isolating the aldehydes of glycerol phosphatides from both horse and beef heart via their dimethyl acetals (58), mixtures were obtained which consisted of C_{14}-, C_{16}-, and C_{18}-aldehydes. Among the unsaturated aldehydes, which occurred only in small amounts, Δ^{9}-octadecenal was found.

Carbohydrates.—The carbohydrate moiety of cerebrosides isolated from the spleen of two cases of Gaucher's disease was identified as glucose by fermentation and spectrographic methods (59). L-arabinose and D-galactose, and galactose, glucose, and glucosamine were reported as constituents of an inositol-containing phosphatide of groundnuts (46) and of a glycolipide isolated from the stroma of bovine red blood cells (49), respectively.

Bases.—Evidence for the occurrence of a new nitrogenous base in phosphatides has been obtained by Ellman & Mitchell (60). The base was isolated from crude cephalin of *Neurospora crassa* by treating the cephalin fraction with dinitrofluorobenzene, hydrolyzing the dinitrophenyl derivatives, and separating the dinitrophenyl-amines by chromatography on silicic acid. The new base was identified as 1-amino-2-methyl-2-propanol. A nitrogenous base not previously observed in inositol lipides of corn and soybean, and which differed from ethanolamine, was isolated by Carter *et al.* (61). The base was found to be identical with the basic component of fungus cerebrin to which Reindel *et al.* (62) had assigned the structure of a 1,2,4-trihydroxy-3-aminoeicosane. The degradation products of cerebrin and its N-benzoyl derivative by oxidation with periodate indicate, however, that the base is a 1,3,4-trihydroxy-2-aminooctadecane, and that the amino carbon atom has the D-configuration with respect to the terminal carbon atom.

Phosphatide intermediates.—The presence of GPC, PC, and sulfurylcholine in aqueous extracts of beef pancreas, lamb liver, and rat seminal vesicles was demonstrated by Diament (63, 64). Of the three constituents the GPC occurs most abundantly. The occurrence of PC in protein-free extracts of beef pancreas was demonstrated also by Greenbaum, Schmidt & Thannhauser (65). In both instances, however, the identification of PC was based on indirect evidence only. GPC was found also in the liver of rabbit, pig, and foetal sheep (8). The occurrence of free ethanolamine phosphate in brain, suggested by the work of Stone (66) and Awapara *et al.*, (67) has been confirmed by Ansell & Dawson (68) by two-dimensional chromatography of the trichloroacetic acid and tungstic acid extracts of whole rat brain. A relatively large ninhydrin-positive spot which proved to be highly resistant to acid and alkaline hydrolysis was identified as ethanolamine phosphate.

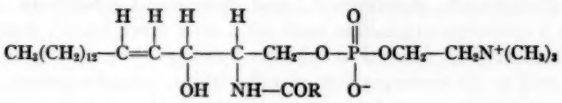
Structure

Lecithins.—An interesting although somewhat involved method for determining the configuration of lecithins has been reported by Long & Maguire (69). The method, which is restricted to the partially unsaturated lecithins, consists of (*a*) incubation of the lecithin with phospholipase-C to remove the PC moiety, (*b*) reduction of the unsaturated diglyceride, (*c*) phosphorylation of the saturated diglyceride by the method of Baer (70) employing diphenylphosphoryl chloride, (*d*) removal of the protective phenyl groups by catalytic hydrogenolysis, and (*e*) saponification of the phosphatidic acid. The saponification of the phosphatidic acid, as Baer & Kates have shown (71), proceeds without migration and yields optically pure α-glycerophosphoric acid. The steric relationship of the enantiomers of the latter to D- and L-glyceraldehyde has been established by Fischer & Baer (72) and forms the basis for all stereochemical assignments in the glycerolphosphatide field. On applying their method to egg lecithin, Long & Maguire confirmed the L-α-structure assigned to this compound four years earlier by Baer & Kates (35) on the basis of the identity of the specific rotations of hydrogenated egg

lecithin [$[\alpha]_D$+6.0° (73)] and synthetic L-α-(distearoyl) lecithin [$[\alpha]_D$+6.1° (35)]. The elucidation of the configuration of egg lecithin by Baer & Kates, it seems, has escaped the attention of Long & Maguire although it has been mentioned in two reviews (74, 75).

The structure of monopalmitoyl lecithin (lysolecithin), obtained by the action of lecithinase-A on lecithin, was determined by Hanahan (76) by oxidation of the lysolecithin with permanganate, and acid hydrolysis of the oxidation product. The formation of glyceric acid-3-phosphoric acid in good yields proved conclusively that the unesterified alcohol group of the lysolecithin is in the alpha position and that the fatty acid ester linkage of the primary alcohol in the intact lecithin is the site of attack by lecithinase-A. Hanahan's lysolecithin structure was confirmed by Long & Penny (77).

Cerebrosides.—By analogy with lecithin the structure of sphingomyelin has been assumed (78) to be as shown by Formula I. The attachment of



FORMULA I. Sphingomyelin.

PC to the primary hydroxyl group of sphinogosine has now been confirmed in a series of investigations by Stotz and co-workers (79, 80, 81). It had been shown that the infrared spectra of 1,4-substituted 2-butenes, regardless of the nature of substituents, are characterized by a strong absorption maximum near 10.3 μ (970 cm^{-1}) if the groups of the double bond have the *trans* configuration. Hence the presence or absence of this *trans* peak may serve as a reliable criterion on which to base a decision pertaining to the geometry of the double bond of sphingosine. A study of the infrared spectra of sphingosine, sphingosine sulfate, and triacetyl sphingosine by Mislow (82) revealed that all three substances possess well defined *trans* peaks near 10.3 μ. Thus, the double bond in sphingosine and its cerebroside precursors has the *trans* configuration. The same criterion served Marinetti & Stotz (83) to establish the *trans* configuration for the double bond of sphingomyelin and N-lignocerylsphingosine. Fodor & Kiss (84), making use of the rule of syncrystallization of Bruni, were able to confirm the *trans* configuration of the double bond in sphingosine.

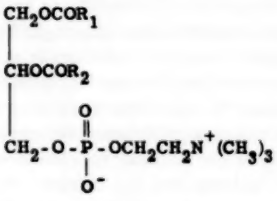
In 1950 Nakayama (85) presented evidence that in phrenosine (cerebron) galactose is attached to the terminal carbon atom of sphingosine. In an inquiry into the validity of some of Nakayama's evidence Carter & Greenwood (86) reinvestigated the structure of phrenosine. Somewhat earlier Carter *et al.* (87, 88) had made the observation that triacetylsphingosine and 3-O-methylsphingosine, on reduction in the presence of an active platinum catalyst, undergo hydrogenolysis of the allylic carbon-oxygen bond in the 3-position, yielding acetic acid or methanol, respectively, and a derivative of sphingosine. By analogy it was, therefore, to be expected that the hydro-

genolysis of acetylated cerebrosides, substituted at C-3 by galactose, would yield free galactose and sphingosine derivatives, whereas cerebrosides substituted by galactose at the terminal carbon atom would yield acetic acid and galactosidosphingosine. The catalytic reduction of hexaacetylphrenosine yielded acetic acid but no reducing sugar, thus confirming the contention of Nakayama, that galactose is attached to the terminal carbon atom of sphingosine.

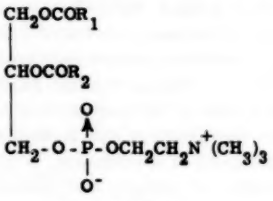
Synthesis

Lecithins.—The procedure of Baer & Kates (35) for the synthesis of the enantiomeric forms of fully saturated α-lecithins has been simplified considerably. In the modified procedure (89) the cumbersome isolation of the phenyllecithin via its reinecke salt is avoided by the use of appropriate solvents for the separation of the phosphorylation mixture.

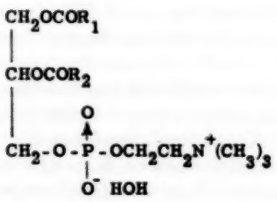
The dimyristoyl-, dipalmitoyl-, and distearoyl-L-α-lecithins were obtained in a distinctly crystalline state by a slow precipitation from chloroform with ether (90). Analytical evidence indicates that the crystalline lecithins, as well as the corresponding glycollecithins, actually possess, in addition to the elements shown by *zwitterion* Formula II, one mole-equivalent of the elements of water (H, OH). The inability to reconcile this fact with certain modern theoretical formulations of the lecithin molecule has led Howton



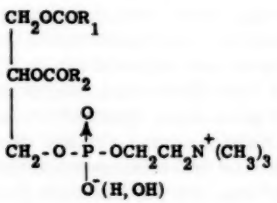
Formula II



Formula III



Formula IV



Formula V

Structure of Lecithin

(91) to infer that they constitute "water of crystallization" and that the structure of the crystalline lecithins is best expressed by the *zwitterion* Formula II. Two modifications (III, IV) of *zwitterion* Formula II, showing a coordinate bond between the phosphorus and oxygen atom were suggested by Devor (92). Formula IV implies that the elements causing the dispute are present as water of crystallization, an assumption that is unsupported by any evidence. It may therefore be advisable to indicate the presence of these elements as shown by Formula V and thus avoid any commitments at the present time as to their nature.

A determination of the molecular weight of synthetic L-α-(dipalmitoyl) lecithin in methanol by the vapour pressure method revealed that at low concentrations (0.9 per cent) it is close to the theoretical value of the monomer, and that even at a comparatively high concentration (4.7 per cent) the lecithin is not associated excessively (89).

A new class of synthetic phosphatides, the glycollecithins, and a generally applicable method for their synthesis, have been reported by Baer (93). The new phosphatides, which can be regarded as analogues of both lecithins and lysolecithins, differ from these substances in that they contain glycol instead of glycerol. The synthesis of the glycollecithins follows in general the procedure of Baer & Kates (35) for the synthesis of α-lecithins, except that monoacylglycols are used as starting materials. The glycollecithins resemble the glycerollecithins in their ability to form alcohol-insoluble cadmium chloride addition compounds containing two moles of lecithin per three moles of cadmium chloride, but differ in that they form water-insoluble reineckates. The composition of these reineckates, however, is unusual since the ratio of glycollecithin to reineckate is 2:1. The glycollecithins, which are obtained readily in a crystalline state, are highly soluble in water and possess strong hemolytic activity.

Phosphatidyl serine.—The synthesis of a fully saturated phosphatidyl serine was accomplished by Baer & Maurukas (37). The method involves three steps (see Fig. 1): (*a*) phosphorylation of an α,β-diglyceride by means of phenylphosphoryl dichloride and pyridine, (*b*) esterification of the resulting diacylglycerylphenylphosphoryl chloride with N-carbobenzoxy serine benzyl ester, and (*c*) removal of the three protective groups by hydrogenolysis with a mixture of platinum and palladium as catalyst. By selecting the appropriate enantiomeric forms of the diglyceride and serine, the phosphatidyl serine can be obtained in any one of its four possible stereoisomers. The synthetic L-α-(distearoyl)phosphatidyl-L-serine was found to be identical with the reduction product of phosphatidyl-L-serine of ox brain. The phosphatidyl moiety of natural phosphatidyl serine thus possesses the α-structure and L-configuration. The phosphatidyl serines constitute the fourth class of naturally occurring glycerolphosphatides for which the α-structure and L-configuration have been established by the Toronto school.

Plasmalogen.—The synthesis of racemic plasmalogen was carried out by Egerton & Malkin (94) in the following manner: α-monolaurin was condensed

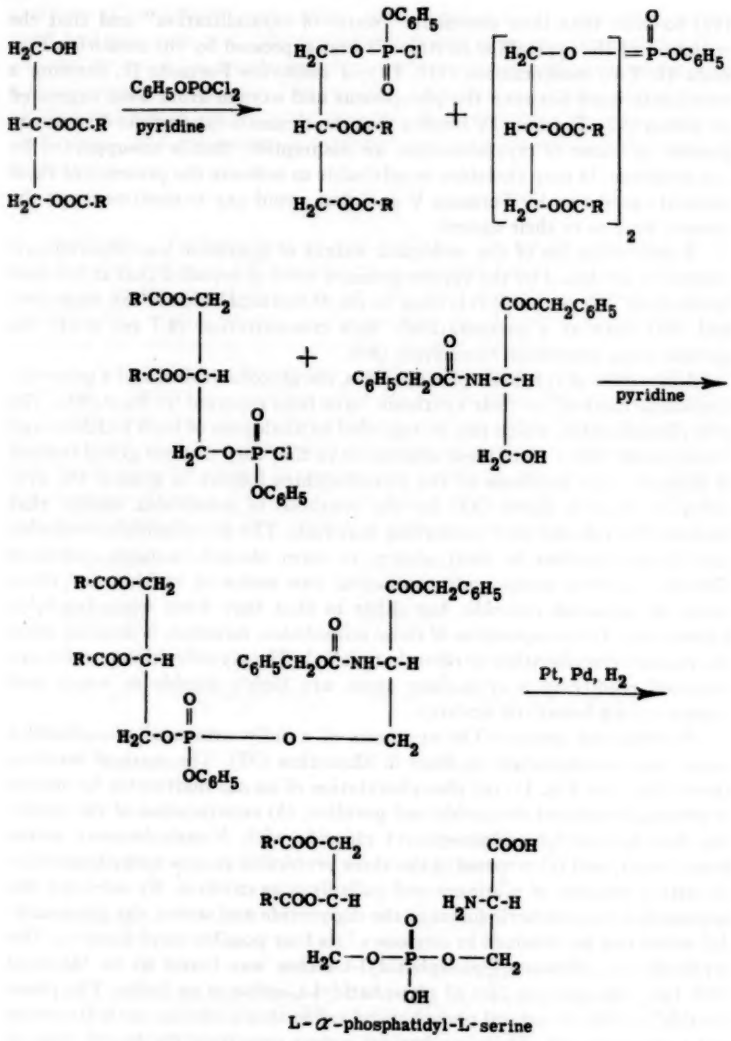


FIG. 1. Synthesis of phosphatidyl serine.

with hexadecanal and the lauroyl group was removed by alkaline hydrolysis. The 2,3-O-hexadecyliden glycerol was phosphorylated by means of phosphorus oxychloride and the resulting plasmalogenoyl dichloride was con-

densed with 2'-hydroxyethylphthalimide. The removal of the phthaloyl group with aqueous hydrazine yielded a crystalline product which analyzed correctly for plasmalogen.

Phosphatidic acids.—Verkade & Uhlenbroek (95, 96), using the method of Baer (70), synthesized the racemic 1,2-distearoyl-, 1,2-dipalmitoyl-, 1-stearoyl-2-palmitoyl-, and 1-palmitoyl-2-stearoyl-3-glycerophosphoric acids.

The dimethyl esters of the enantiomeric forms of fully saturated α-phosphatidic acids have recently acquired interest as reference compounds in the elucidation of the structure and configuration of the phosphatidyl moiety of naturally occurring cephalins and phosphatidyl serines by a degradation procedure using diazomethane (38). The dimethyl esters of several phosphatidic acids most likely to be encountered as cleavage products of the hydrogenated forms of natural cephalins and phosphatidyl serines, namely distearoyl-, dipalmitoyl-, and dimyristoyl-L-α-glycerophosphoric acid, were obtained by methylation of the corresponding phosphatidic acids (70) with diazomethane (38).

In an earlier investigation Faure & Legault-Démare (97) had shown that lecithins in nonpolar solutions (benzene, isoamyl ether) exist in the micellar state, whereas in polar solutions (methanol, ethanol, acetic acid) they assume the molecular state. Extending these studies to the inorganic and organic salts of phosphatidic acids of wheat germ and heart muscle, they (98) found that in nonpolar solutions the sodium and potassium salts of these phosphatidic acids assume a micellar condition, whereas their quinine and tetramethyl ammonium salts exist in the molecular state. In polar solvents the inorganic as well as organic salts of both phosphatidic acids, however, assume the molecular state.

Bisphosphatidic acids.—In view of the current interest in the phosphatidic acids as cardiolipin substitutes in the serodiagnosis of syphylis, a general procedure for the synthesis of fully saturated α-bis-(glycerol)-phosphatidic acids in either of the two enantiomeric forms was developed by Baer (99). The bis-phosphatidic acids are obtained by the phosphorylation of two moles of an α,β-diglyceride with one mole of monophenylphosphoryl dichloride in the presence of pyridine, and subsequent removal of the protective phenyl group by hydrogenolysis. The tetramyristoyl bis-(L-α-glyceryl)phosphoric acid was found to possess cardiolipin-like activity (100). The first two representatives of bis-(glycol)-phosphatidic acids (101), namely the distearoyl and dipalmitoyl bis-(glycol)-phosphoric acids, were obtained by phosphorylation of monostearoyl or monopalmitoyl glycol with phenylphosphoryl dichloride and pyridine, and subsequent removal of the phenyl group by catalytic hydrogenolysis.

Glycerylphosphorylethanolamine.—To establish the configuration of cephalins and plasmalogens, α-glycerylphosphorylethanolamine (GPE), which is a moiety of both phosphatides and also occurs in the free state in the liver of pigs, rabbits, foetal sheep, and rats (102), as well as in the liver, spleen, kidney, and brain of oxen (103), was synthesized by Baer & Stancer (104).

The L-isomer was obtained by phosphorylation of D-acetone glycerol with phenylphosphoryl dichloride and quinoline, esterification of the resulting acetoneglycerylphenylphosphoryl chloride with N-carbobenzoxyethanolamine, and removal of the protective carbobenzoxy, phenyl, and acetone groups by catalytic hydrogenolysis and mild acid hydrolysis, respectively. The melting point (86°–87°C.) and specific rotation (−2.9°) of the synthetic product agreed well with those reported by Feulgen & Bersin (86°–87°C.) (128) and by Thannhauser, Boncoddo & Schmidt (−3.2°) (129) for natural GPE and thus made it possible for Baer & Stancer (104) to assign the L-configuration to natural GPE and plasmalogen. Arnold's alleged synthesis of β-glycerylphosphorylcholine (105) was reinvestigated by Režek (106) who confirmed Aloisi & Buffa's (107) finding that the procedure of Arnold yields choline β-glycerophosphate and not as claimed, β-glycerylphosphorylcholine.

Dihydrosphingosine.—In an earlier paper Grob *et al.* (108) described the synthesis of one of the two racemic forms of 2-amino-octadecane-1,3-diol (m.p. 99.5°-100.5°C.) and reported that the infrared spectrum of its triacetyl derivative was found to be identical with that of the corresponding derivative of natural dihydrosphingosine. The resolution of the synthetic 2-amino-octadecane-1,3-diol by means of L-glutamic acid (109) gave in good yields a dihydrosphingosine whose melting point (109°C.) and optical activity ($[\alpha]_D$ −13.5°) agreed well with those reported by Seydel (110) for natural dihydrosphingosine.

Improvements in the procedure for the synthesis of racemic 2-amino-octadecane-1,3-diols by the method of Grob, Jenny & Utzinger (108) were reported by Egerton, Gregory & Malkin (111). According to Malkin *et al.* the condensation of hexadecanal with 2-nitroethanol by sodium methoxide instead of sodium hydroxide, and the reduction of the 2-nitro-octadecane-1,3-diols in the presence of platinum oxide (Adams' catalyst) instead of Raney nickel, results in a greatly improved yield of the mixture of racemic 2-amino-octadecane-1,3-diols.

Further syntheses of racemic 2-amino-octadecane-1,3-diols were carried out by Fisher (112), and Proštenik & Stanačev (113). Since none of the known synthetic methods provided evidence as to the stereochemistry of the natural product, Carter, Shapiro & Harrison (114) devised a method for the synthesis of the racemic forms of *threo-* and *erythro-*1,3-dihydroxy-2-amino-octadecane from the *threo-* and *erythro-*α-amino-β-hydroxystearic acids. The determination of the configuration of natural dihydrosphingosine as the *erythro-*form was made possible by the fact that only the *erythro* forms yield tribenzoyl derivatives (m.p. 144°–145°C.). The resolution of the synthetic *erythro-*1,3-dihydroxy-2-aminooctadecane via the glutamic acid salts by Carter & Shapiro (115) yielded two optical isomers, one of which proved to be identical with the natural base. On the basis of these data and in conjunction with a previous determination of the configuration of the amino carbon (88), Carter *et al.* assigned the *erythro-*D-1,3-dihydroxy-2-aminooctadecane structure to natural dihydrosphingosine.

A stereospecific synthesis of *threo*-dihydrosphingosine has been reported by Jenny & Grob (116). This compound is of interest since it may be identical with one of the two reduction products of sphingosine described by Seydel (110).

Sphingine.—Sunko & Proštenik (117) described a new synthesis of racemic sphingine and its resolution into the optically active forms by the following method: Heptadecanoic acid was treated with thionyl chloride and the resulting acid chloride (I) was converted into 1-diazo-2-octadecanone (II) by means of diazomethane. On treating compound II with glacial acetic acid, 1-acetoxy-2-octadecanone (III) was obtained. The hydrogenation of III in methanolic-benzylamine in the presence of Adams' catalyst (IV), and subsequent hydrolysis, gave 1-hydroxy-2-benzylaminooctadecane (V), which on debenzylation with palladium on barium sulphate catalyst yielded sphingine (VI). The sphingine was also obtained by reducing the oxime of compound III with lithium aluminum hydride. The resolution of the racemic sphingine was effected via the L(+)glutamic acid salts. The more soluble salt on decomposition with sodium carbonate gave a levorotatory sphingine ($[\alpha]_D$-4.92°, m.p. 84°–86°C.) which proved to be identical with natural D(−)sphingine ($[\alpha]_D$–5.5°, m.p. 84–89°C.) (88).

Chemical Hydrolysis

Baer & Kates, in a study of the hydrolysis of synthetic L-α-glycerylphosphorylcholine (118) and L-α-lecithins (71) by acid or alkali, had demonstrated unambiguously that the degradation of these phosphatides is accomplished by a reversible migration of phosphoric acid. In a continuation of these studies and by use of synthetic cephalins and GPE of known constitution (α) and configuration (L), Baer, Stancer & Korman (119) found that the hydrolysis of these substances both by acid or alkali is likewise accompanied by a reversible migration of phosphoric acid. The composition of the hydrolysis products suggested that the acid and alkaline hydrolyses proceed via cyclic orthoesters and that the chemical hydrolysis of both lecithins and cephalins follows similar reaction mechanisms.

By use of sheep brain dehydrogenase for the estimation of L-α-glycerophosphoric acid, Long & Maguire (120) investigated the composition of the glycerophosphoric acid mixtures obtained by alkaline hydrolysis of soybean lecithin, egg lecithin, beef heart lecithin, and ox brain cephalin. On finding that the alkaline hydrolysis of the natural phosphatides and authentic L-α-phosphatides gave mixtures of glycerophosphoric acids which contained similar proportions of L-α-, D-α-, and β-glycerophosphoric acids, Long & Maguire concluded that the lecithins of soybean, egg yolk, and beef heart, and the cephalin of ox brain stereochemically belong to the L-series. This conclusion is in agreement with earlier assignments of the L-configuration to naturally occurring glycerolphosphatides by Baer and associates.

Schmidt, Bessman & Thannhauser (121) report that the ester bond from ethanolamine to phosphoric acid is much more labile in GPE than in phosphorylethanolamine, both in acid and alkali, and that the ethanolamine

of GPE is released without the formation of phosphoric acid. Several years ago Baer & Kates (122), while investigating the mechanism of the alkaline hydrolysis (0.2 *N* NaOH) of synthetic L-α-(dipalmitoyl) lecithin (0.02 *M*) in aqueous methanol (90 per cent) and carbon tetrachloride at 37°C., had shown that the fatty acids are liberated at a considerably higher rate ($k = 1.0$ hr.$^{-1}$) than choline ($k = 6.9 \times 10^{-3}$ hr.$^{-1}$), and that at the end of 4 hr., under the specified conditions, approximately 80 per cent of GPC is present in the hydrolysis mixture. Diament (63) has now described procedures by means of which the GPC can be isolated in yields of approximately 73 per cent of theory.

Verkade & Uhlenbroek (123), in a study of the alkaline hydrolysis of racemic α- and of β-glycerolphosphatidic acids confirmed the observation of Baer & Kates (122) that the liberation of the fatty acids takes place without migration of the phosphoric acid. They observed, however, that if esters of α-glycerolphosphatidic acids are treated with alkali, phosphoric acid migration occurs with the formation of β-glycerophosphoric acid. Since it can be assumed that the liberation of the fatty acids precedes the hydrolysis of the glycerophosphoric acid ester, and the latter, as Bailly & Gaumé have shown, undergoes rearrangement of phosphoric acid on hydrolysis with alkali (124), the formation of β-glycerophosphoric acid was to be expected.

An interesting procedure for the dephosphorylation of cephalins and lecithins has been reported by Malkin *et al.* (125). The PC or PE moiety is replaced by acetic acid, the exchange taking place in a boiling mixture of acetic anhydride and glacial acetic acid (1:4, v/v). The procedure, however, is accompanied by acetyl migration and thus does not lend itself to a determination of the structure of lecithins or cephalins.

While trying to prepare a methyl ester of L-α-(distearoyl) phosphatidyl-L-serine by means of diazomethane, it was observed by Baer & Maurukas (38) that this reagent causes a cleavage of the ester bond linking the phosphatidic acid to serine and leads to the formation in good yields of L-α-(distearoyl)-phosphatidic acid dimethyl ester. This reaction, it was found, is also given by cephalins but not by lecithins. The unique and extremely useful feature of the reaction, for which the name diazometholysis was proposed, is that it takes place without structural or configurational changes in the phosphatidic acid moiety. Since the phosphatidic acid dimethyl esters obtained by diazometholysis of the natural phosphatides can be compared with synthetic phosphatidic acid dimethyl esters of known constitution and configuration, the new procedure offers a simple and unambiguous method for the elucidation of the structure and configuration of the glycerophosphoric acid moieties of natural cephalins and phosphatidyl serines. The application of the reaction to the phosphatidyl serine of ox brain confirmed the α-structure and L-configuration of its glycerophosphoric acid moiety which had been established by comparison of the reduction product of natural phosphatidyl serine with synthetic L-α-(distearoyl) phosphatidyl-L-serine (37).

The question as to whether *glycérophosphatogène* is an artefact of the

alkaline hydrolysis of lecithins is receiving further study in Fleury's laboratory.[3]

X-Ray Diffractions

Finean (126), in a study of the x-ray diffraction patterns of phosphatides at various temperatures, observed changes in the patterns that varied considerably with the type of phosphatide. In general it would seem that the long spacings of the phosphatide tend to decrease with rise in temperature, the decrease being associated with tilting of the long axes of the molecules with respect to the plane of the bimolecular leaflet. In the case of the cephalins, this tilting appears to take place in steps, thus giving rise to a set of distinct polymers. With lecithins and sphingomyelin the process of tilting seems to be more continuous, although these two show distinct polymorphic forms at certain stages of heating. The difference in behavior may be associated with differences in the nature of the end groups. X-ray diffraction patterns obtained at temperatures above the sintering point of the phosphatides should, however, be considered with great caution since the sintered lecithins and cephalins may have undergone irreversible chemical transformations. X-ray diffraction patterns were also reported for the synthetic dimyristoyl-, dipalmitoyl-, and distearoyl-L-α-cephalins (127), stearoyl glycollecithin (101), and L-α-glycerylphosphorylethanolamine (104).

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METABOLISM OF COMPLEX LIPIDES[1,2]

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The present review will deal with selected topics in lipide metabolism—in particular those aspects of the metabolism of phosphatides that have not received a reviewer's attention since the article in this series by Artom two years ago (1). Although an attempt has been made to review the metabolism of all complex lipides, the paucity of data on lipides, other than phosphatides, has restricted the scope of this article primarily to the phosphorus-containing lipides. For the chemistry and distribution of cerebrosides the recent review by Celmer & Carter (2) may be consulted.

The increased employment of a more precise nomenclature for the complex lipides is noteworthy. Unfortunately, in most biological studies, the questionable homogeneity and purity of the final lipide extracts prevent the use of a chemically acceptable terminology. Thus we must continue to use the more ambiguous terms "lecithin," "cephalin," and "phosphatide" (or "phospholipide").

Absorption of Phosphatides

The absorption of phosphatides from the digestive tract has been studied by Bloom *et al.* (3) who found that rats fed C^{14}-labeled phosphatide, may absorb as much as 25 per cent of this lipide without hydrolysis of the molecule. The experiments of Leonhardi *et al.* (4) similarly indicate that fed phosphatides reach the blood of rats by some means other than hydrolysis and subsequent resynthesis, since the relative amount of radioactive phosphatide of rat skin was greater after feeding P^{32}-labeled phosphatides than after radioactive phosphate. Further indirect evidence that some phosphatides are absorbed intact can be gleaned from the experiments of Popper *et al.* (5) who showed that in rats trimethylamine excretion in the urine is greater upon feeding free choline than after administration of equivalent amounts of lecithin.

Fed phosphatides, like other lipides, are absorbed by way of the lymphatics. The source of lymph phosphatides has been studied by Reiser *et al.* (6) who observed that in rats fed triglycerides labeled with C^{14}-glycerol and conjugated fatty acids about half the lymph phosphatides were derived from free fatty acid and the other half from absorbed monoglycerides.

Reiser *et al.* studied the role of intestinal phosphatides in fat absorption. A preliminary report (7) furnished evidence that phosphatides could be intermediates in fat absorption, but further investigation (8) indicated that

[1] Pertinent literature available before November, 1954 is included in this article.

[2] The following abbreviations are used: ATP for adenosinetriphosphate; DCA for desoxycorticosterone acetate.

ingested labeled glycerol underwent greater dilution in the phosphatides of mucosa and lymph than in lymph triglycerides, thus suggesting that mucosal triglycerides are the precursors of phospholipides rather than the reverse. This question was also studied by Favarger & Gerlach (9, 10) who showed that the uptake of labeled palmitic or stearic acid by intestinal phospholipides was doubled or tripled when the deuterium-labeled palmitin or stearin which was fed was diluted with lard. Apparently the epithelial cells of the intestine synthesize phosphatides more rapidly when both saturated and unsaturated fatty acids are available. Borgström (11) demonstrated that long-chain fatty acids which are absorbed by way of the lymphatics are incorporated into intestinal phospholipides to a greater extent than the short chain fatty acids which appear in the portal vein. Thus it seems that the rate at which ingested fat contributes fatty acid for phospholipide synthesis is related to the digestibility (9) and pathway of absorption of the lipide (11). Favarger & Gerlach (9, 10) reject the hypothesis that phosphatides are intermediates in fat absorption on the basis of their observation that the isotope concentration of the phosphatides in different parts of the small intestine was practically the same, whereas the isotope concentration of triglycerides varied considerably. In a similar study, free glycerol and glycerophosphate were eliminated as possible precursors of intestinal phospholipides [Buensod *et al.* (12)]. Borgström (13) also concluded from feeding C^{14}-palmitic acid to rats that all intestinal phospholipides do not function as intermediates in fat absorption. None of the experiments exclude the possible participation in fat absorption of a small fraction of the intestinal phosphatides, but it does not appear profitable to maintain such an hypothesis in the absence of convincing evidence.

In the study of the partition of C^{14}-stearic and palmitic acid between the lipides of rat intestinal lymph, Borgström (14) observed that about 90 per cent of the label appeared in the triglycerides and as much as 10 per cent in the phosphatide fraction. These findings indicate that during fat absorption the intestinal wall supplies an important part of the plasma phospholipide. Favarger (9) has pointed out, however, that the extensive labeling of phosphatides by saturated fatty acids in Borgström's experiments may be attributable to the relatively small amounts of saturated fatty acids present in the corn oil diluent, resulting in the selective uptake of the label. After feeding labeled stearic acid to rats, the specific activities of plasma phospholipide were higher than those of plasma triglyceride [Bergström *et al.* (15)]. Apparently the lymph triglycerides are selectively removed from the blood stream whereas the plasma and lymph phospholipides mix more completely before their eventual utilization. The phospholipide content of rat-lymph and rat-serum chylomicrons was determined by Laurell (16) and by Byers & Friedman (17). The role of bile in the metabolism of intestinal phospholipides was studied by Borgström (18) and by Pessoa *et al.* (19). The former showed that the formation of C^{14}-labeled phospholipides after fat feeding is not altered in rats deprived of bile; the latter noted that, in con-

trast to normal animals, fecal phospholipide excretion after fat feeding increases markedly in bile fistula dogs.

Blood Phosphatides

Acetal phosphatides.—The study of acetal phosphatides has not gained the attention of many biochemists, and the data obtained in this field have not yet stood the test of comparison and contrast. Something may, therefore, be gained by presenting the recent achievements in this area under a single heading. The observation of Taubert & Schäfer (20) that the serum acetal phosphatides of diabetics are depressed was not confirmed by Leupold & Büttner (21). These authors found the serum plasmalogen levels of women to be significantly higher than those of men (22). Voit & Seckfort (23), however, did not demonstrate any difference in the plasmalogen levels of 100 normal men and women. These investigators noted the absence of significant alterations in plasmalogen concentrations during the menstrual cycle or after the menopause. Seckfort (24) has reviewed the relation of plasmalogen level to disease and noted that the level depends more on the nutritional state of the patient than on any specific disease. Very high plasmalogen levels were, however, observed in obstructive jaundice. In hepatitis Thiele (25) observed depressed serum plasmalogens, whereas cirrhotics showed normal values. Thiele suggests that the liver is not the source of serum acetal phosphatides. After feeding 50 gm. of olive oil, which contains very little acetal phosphatides, the serum acetal phosphatide levels of human subjects remained unchanged even though other phosphatides increased in concentration [Thiele (26)].

The administration of acetylcholine and physostigmine decreased serum plasmalogen as much as 20 per cent in 1 to 2 hr., and a possible parasympathetic control of plasmalogen metabolism should be considered [Schäfer & Höreth (27)]. Intense stress in the form of scalding, x-ray, or nitrogen mustard markedly increased serum plasmalogen concentrations of rats whereas the administration of histamine or exposure of the animals to cold did not result in altered plasmalogen levels [Thiele (28)]. Chemical analysis of the whole animal showed that the additional serum plasmalogen was derived from an increased synthesis of this material rather than from a transfer of tissue phosphatide to the bloodstream. Serum plasmalogens, just as other phosphatides, occur as part of lipoprotein complexes. Leupold *et al.* (29) showed by alcohol fractionation and paper electrophoresis that acetal phosphatides of human serum occur primarily in the alpha- and beta-globulin fractions. In the erythrocyte, all the acetal phosphatide is found in the stroma where it comprises a greater fraction of the total phosphatides than in serum. Acetals have been demonstrated as minor constituents of all tissues except muscle and nerve [Korey & Wittenberg (30)].

Diet and serum phosphatides.—Not long ago the dietary modifications of serum lipide levels were studied for their own sake or to increase our understanding of the relatively rare lipometabolic diseases. The study of serum

phospholipides recently received new impetus from the possible relation of serum lipides to atherosclerosis. Morrison (31), for example, investigated the effect of lipotropic agents on serum lipide levels. He found that patients, who for years lived on a low-fat diet, showed an additional lowering of the cholesterol-phospholipide ratio when betaine was added to the diet. Later work indicated that this ratio was also lowered by a combination of the low-fat diet with a mixture of lipotropic agents [Morrison (31)]. The lack of statistical evaluation of these results as well as of those of others and the absence of information on the content of lipotropic agents in the basal diets make it difficult to interpret the conflicting claims about the effects of various lipotropic agents. Goldbloom *et al.* (32), Greenberg & Bruger (33), and De Wind *et al.* (34) could find no effect of lipotropic agents on serum cholesterol or phospholipides in patients with generalized atherosclerosis or diabetes. Duff & Meissner (35) met with similar failure in rabbits on a high cholesterol diet. On the other hand, the studies of Dotti *et al.* (36) and Felch *et al.* (37) indicate that inositol depresses serum cholesterol and phospholipide in cholesterol-fed rabbits and in survivors of myocardial infarcts, respectively. In a careful study of 13 patients McKibbin & Brewer (38) failed to find an increase in plasma inositol in response to inositol feeding and found only a slight if any increase in total plasma phosphatides.

Chlortetracycline (aureomycin) has been mentioned as a choline-sparing material, and the studies of Kaplan *et al.* (39) support this hypothesis. The feeding of this antibiotic raised the characteristically low serum phospholipide and cholesterol levels of pancreatic-duct ligated dogs, though the effect diminished after several weeks of chlortetracycline feeding. Vitamin B_{12} and folic acid exhibited a synergistic action with chlortetracycline, although administered by themselves they were without effect on blood lipide levels. Feinberg *et al.* (40) observed that the feeding of ethionine to normal dogs caused a fatty liver and a marked decrease in all plasma lipides without changing the cholesterol-phospholipide ratio. It has been shown that the feeding of soybean phosphatides to hypercholesterolemic patients on a high-fat diet lowered serum cholesterol levels [Pottenger & Krohn (41)]. Moses (42) on the other hand did not observe changes in blood lipide levels of hypercholesterolemic rabbits fed inositol phosphatide supplements. A successful method of lowering serum lipides appears to be the feeding of a brain powder to cockerels on a high cholesterol diet [Jones *et al.* (43); Gordon *et al.* (44)]. These workers observed that a cerebroside fraction of brain lowers serum phospholipides and the cholesterol-phospholipide ratio. Similarly the isocaloric substitution of plant fat for animal fat in the diet of patients may result in a marked lowering of cholesterol and phospholipide levels [Kinsell *et al.* (45, 46); Cochrane *et al.* (47)]. This effect is not attributable to the absence of cholesterol from the plant fat since addition of as much as 60 gm. of cholesterol did not restore the lipides to their previous levels. These findings have been confirmed by Ahrens *et al.* (48) who administered about 50 per cent fat to six subjects over a period of four months. Replacement of

animal fat by plant fat lowered the free and total serum cholesterol to a greater degree than it lowered the serum phospholipides. Similar findings are reported by Beveridge *et al.* (49). Blood lipide studies on vegetarians also indicate that cholesterol levels appear more closely correlated to the intake of animal fat than to the amount of total fat in the diet [Hardinge & Stare (50)].

Hormones and serum phosphatides.—The study of serum lipide levels in endocrine disorders has been complicated by the effects of hormones on blood volume and the state of hydration of the subject. Thus, alterations in serum lipide levels may be produced by an abnormal fat metabolism or may merely be the result of a passive dilution of the serum solids. To distinguish between these possibilities several investigators have related serum lipide concentrations to the level of serum proteins; others have measured plasma volumes by some dilution technique. Even though the cholesterol-phospholipide ratio measures metabolic alterations without the disturbing effects of hydration, the cholesterol and phospholipide levels frequently change proportionally so that little or no information is obtained from the ratio of these two lipides. For example, in 10 out of 14 diabetic patients with glomerulosclerosis, serum phospholipides were elevated, but only a few of these patients showed elevated cholesterol-phospholipide ratios [Engelberg *et al.* (51)]. Petersen (52) observed elevated serum phospholipides in young diabetics but not in older ones, and with normal phospholipide-cholesterol ratios in all. Harris *et al.* (53) observed a moderate increase in serum phospholipides and in the ratio of free cholesterol to phospholipide in diabetic acidosis. In alloxan diabetic rats the serum phosphatides were not elevated as much as were cholesterol and total fatty acids [Cagan *et al.* (54)]. Fasoli *et al.* (55) studied the lipide fractions of serum lipoproteins in normal and diabetic dogs. In depancreatized dogs maintained on insulin and with raw pancreas added to their diet the serum phospholipides increased. They increased even further when insulin was withdrawn. It is interesting to note that the dog apparently carries a smaller portion of its serum phospholipide in the beta-lipoprotein fraction than man (55, 56).

The action of estrogens on serum lipides has also received further attention during the last few years. Gertler *et al.* (57) observed that orchiectomy followed by daily doses of diethylstilbesterol increased serum phosphatides and lowered the cholesterol-phosphatide ratio. Eilert (58) made similar observations after the administration of ethinyl estradiol or stilbesterol to women during menopause. Small doses of ethinyl estradiol were found to depress the cholesterol levels of hypercholesterolemic men without affecting the phosphatides [Oliver & Boyd (59)]. Similarly some decrease in serum phosphatide with a more marked decrease in cholesterol-phosphatide ratio was observed in women at a point of the menstrual cycle where the estrogen activity is known to be maximal (60). In cockerels on high cholesterol diets the administration of estrogen elevated plasma phosphatides with a subsequent depression of the cholesterol-phosphatide ratio [Pick *et al.* (61)]. On

the other hand Glass *et al.* (62) failed to observe any effect of physiological doses of estradiol on phosphatides or other lipides of men and postmenopausal women. A lowering of serum phosphatides and other lipides by daily injection of testosterone into diabetic patients was noted by De Wind *et al.* (34).

Myxedematous patients showed not only elevations of cholesterol and phospholipide levels, but all except one also exhibited increased cholesterol-phospholipide ratios (Malmros & Swahn (63)]. Serum phospholipides were also found to be slightly elevated in hyperthyroid individuals [Schulze (64)]. Treatment with propylthiouracil in most cases increased the phospholipide level even more. A transient rise in phospholipide levels was observed in rats 6 hr. after injection of growth hormone [Greenbaum & McLean (65)].

The role of the adrenals in the regulation of serum phosphatide levels has been investigated by Migeon (66), who observed that the administration of cortisone to intact rats increased serum phosphatides but had no effect on the lipide levels of adrenalectomized rats maintained on DCA[3]. Adrenalectomized animals without DCA did not show a significant alteration in serum phosphatide levels. Apparently the dog behaves differently; adrenalectomized dogs maintained on DCA showed marked decreases of plasma phosphatide and cholesterol levels without significant alterations in cholesterol-phospholipide ratios [DiLuzio *et al.* (67)]. The administration of cortisone after DCA withdrawal rapidly restored plasma lipide levels to normal or above normal values. It appears that in the dog cortisone is essential for the maintenance of normal plasma phospholipide and cholesterol levels. The parallelism between the levels of cholesterol and phospholipide over a wide range raises the interesting question whether cholesterol and phospholipide levels are regulated by a similar mechanism, or whether the adrenal hormones act primarily on the protein moiety of the lipoprotein molecule of which cholesterol and phospholipide constitute a fixed portion. These possibilities were also discussed by Feinberg *et al.* (40) who demonstrated that ethionine markedly lowered serum phospholipides and cholesterol concentrations in dogs without alteration of the ratio between the two lipides. A parallelism between cholesterol and phospholipide was not observed after the stress of subjecting a normal dog to a 100 hr. fast [Mann & White (68)]. During this period the plasma cholesterol level fell continuously, but the phospholipides remained constant. Gordon *et al.* (69) studied the effects of cortisone on serum lipides of rabbits. Cortisone increased serum phosphatide and cholesterol levels as did the feeding of cholesterol. However, cortisone plus cholesterol did not exert a cumulative effect on serum phospholipides. Similar experiments of Stumpf & Wilens (70) and Adlersberg *et al.* (71) showed cortisone and cholesterol to act synergistically in this respect. The extremely high levels of serum cholesterol and phospholipide in rabbits receiving cholesterol plus cortisone were diminished somewhat when hyaluronidase was also administered [Wang *et al.* (72)]. The authors suggest that the inhibition of atherogenesis by cortisone, despite the high lipide levels, may be related to the decreased tissue permeability produced by cortisone. In this connection

it is interesting to note, however, that the entry of liver phosphatides into the circulation of adrenalectomized dogs was accelerated by cortisone [Zilversmit *et al.* (73)] rather than diminished, suggesting that in this tissue at least, cortisone did not decrease the permeability to lipide materials. Rabbits submitted to injury by cold exhibited an increase in phosphatide and cholesterol levels without alteration in the ratio of these two lipides [Milch *et al.* (74); Redmond *et al.* (75)]. Stamler *et al.* (76) observed that hydrocortisone and corticotropin produced a cholesterolemia and phospholipemia in cholesterol-fed chicks, but that cortisone did not. Increases in plasma phosphatide levels after whole body x-radiation were observed in rabbits, dogs, mice, guinea pigs, and rats by Entenman *et al.* (77).

Heparin and serum phosphatides.—Even though it is now evident that a lipemia clearing factor modifies the metabolism of serum triglycerides, the effect of this factor on other lipides is poorly understood, and conflicting reports are still appearing in the literature. Bolinger *et al.* (78) and Boyle (79) observed that heparin decreased the serum phospholipide and cholesterol concentrations of hypercholesterolemic patients. A shift in lipide material to the alpha-globulin region prompted the suggestion that heparin accelerates the conversion of free to protein-bound-phospholipide (78). Similarly Treburon, a synthetic heparinoid material which was shown to clear alimentary lipemia [Ackerman & Zilversmit (80)] produced a significant reduction in serum phospholipide and cholesterol levels [Chandler *et al.* (81)]. Sartorius *et al.* (82) report, however, that heparin caused an increase in serum phosphatides of hypertensive patients treated with this drug over a prolonged period of time, whereas Herzstein *et al.* (83) and Soffer & Murray (84) observed no effect of heparin on serum phosphatides. The effects of antiheparin agents have been studied by Bragdon & Havel (85). These workers injected protamine into fasting rats and observed a marked increase in all serum lipide fractions. They found that heparin, on the other hand, did not affect the serum lipide pattern of fasting rats. Levinsky *et al.* (86) observed no change in the rate of serum phosphatide formation in coronary patients and normal controls after injection with heparin, but the variation from patient to patient was considerable.

Phosphatides as part of the lipoprotein complex.—Even though most of the serum phosphatide is protein-bound, the lipide chemist has studied the serum phospholipides as a form of lipide and has considered the protein segment as an incidental entity. However, more recently, with the aid of newer and more powerful techniques attention has swung to the study of the entire lipoprotein complex. By paper electrophoresis of P^{32}- and S^{35}-labeled rat and rabbit serum, the association of lipide and protein was investigated by Maurer (87) and Maurer & Müller (88). These workers found that radioactive phospholipide moved with the serum-albumin fraction if electrophoresis was carried out in a mixture of barbital (Veronal) and serum rather than in pure barbital buffer. These authors concluded that in the native state phosphatides are bound to albumin rather than to serum globulin. However,

the binding of phosphatides to globulins is so well established from chemical fractionation and ultracentrifugation studies, that further work on this controversy is indicated. Even so, these electrophoretic studies show that the bond between phospholipide and protein is very labile. In an ultracentrifugal study of human serum lipoproteins Turner *et al.* (89, 90) have demonstrated that two-thirds to three-fourths of the total serum phosphatide is carried by a lipoprotein devoid of cholesterol which was found to turn over more rapidly than the rest of the serum phosphatide. This interpretation is not easily reconciled with the findings of Kunkel & Bearn (91) who showed that the phosphatide specific activities in α_1-, α_2-, and β-lipoproteins are nearly equal and that phosphatides of the different lipoproteins readily exchange with each other. Similarly Eder *et al.* (92) and Zilversmit *et al.* (93) observed nearly equal specific activities of high-density and low-density lipoproteins of rabbits injected with P^{32}. These authors also noted a rapid *in vitro* exchange of labeled phosphatides between the high-density and low-density lipoproteins.

In an attempt to determine the effect of the protein moiety on the metabolism of serum phosphatides, Zilversmit (94) injected P^{32}-labeled egg yolk lipoproteins into dogs. Lecithin constitutes about 80 per cent of the egg yolk phosphatide and is bound to protein in the form of lipovitellin [Hawke & Lea (95); Van Handel (96)]. The injected "foreign" lipoprotein disappeared from the blood stream with an initial half-time of 6 hr. which compares rather closely to that for native lipoproteins. Furthermore, the tissue distribution of egg yolk phosphatides was similar to that of native plasma phosphatides. Therefore, it appears that the protein portion of the lipoprotein does not exert a marked effect on the metabolism of the lipide moiety, or, more likely, that the egg lipoprotein readily exchanges its phosphatide with the circulating serum proteins. A rather stable phospholipoprotein was found in the serum of laying or stilbesterol-injected birds by Clegg & Hein (97). This fraction was not present in the serum of nonlaying controls.

Turnover of serum phosphatides.—When P^{32} was injected directly into the bone marrow of rats the specific activity of plasma phosphatides was considerably higher than that of liver, suggesting that bone marrow may contribute some phospholipide to the plasma of the rat [Claassen *et al.* (98)]. The hypothesis that these highly radioactive plasma phosphatides are derived from bone marrow was supported by the demonstration of phospholipide synthesis, *in vitro*, by bone marrow [Claassen *et al.* (99)]. In the fasting dog, however, the role of the liver as the main source of plasma phosphatides appears well established. In recent studies Harper *et al.* (100) showed that hepatectomy virtually stopped the synthesis of plasma phospholipides from labeled acetate. However, some exchange between phospholipide of plasma and that of depot fat still occurred in the liverless dogs. The hypothesis that the turnover rate of plasma phosphatides is directly proportional to the concentration of these lipides has been stated by Balfour (101). Supporting evidence has been derived from the studies of Zilversmit *et al.*, showing an

increased turnover and concentration of plasma phosphatides in depancreatized dogs without insulin therapy (102), phlorizinized dogs (103), adrenalectomized dogs maintained on cortisone (73), and in hypercholesterolemic rabbits (104). Paraf *et al.* (105) did not find a decrease in liver lipides of bile-obstructed dogs in which the plasma phosphatides were elevated. These authors suggest that the phospholipemia is, therefore, not the result of hepatic overproduction of phosphatides from other lipide precursors.

Phosphatides in atherosclerosis.—Serum phosphatides have been studied in an effort to decide whether the relative amounts of phosphatide and cholesterol may be useful as an index of atherogenesis. Among those who have found a clear-cut relation between increased serum cholesterol-phospholipide ratios and the presence of some form of atherosclerosis in man, are Morrison (106), Steiner *et al.* (107), Oliver & Boyd (108), and Gertler & Oppenheimer (109). High cholesterol-phospholipide ratios were correlated with greater incidence of coronary atherogenesis in chickens [Pick *et al.* (110); Jones *et al.* (43)] and with atheromatous lesions in rats [Wissler *et al.* (111)] and rabbits [Gordon *et al.* (69)]. Among those who failed to find any relation between cholesterol-phospholipide ratios and the incidence of atherosclerosis are Jackson & Wilkinson (112), Goldbloom & Pomeranze (113), Barbagallo-Sangiorgi & Caiozzo (114), Mendlowitz *et al.* (115), Katz *et al.* (116), Iannaccone *et al.* (117, 118), Stumpf & Wilens (70), Paterson *et al.* (119), and Little *et al.* (120).

A different approach to the atherosclerosis problem has been taken by Horlick (121) who observed that when lecithinase was allowed to act on serum lipoproteins, serums of older individuals developed turbidities faster than those of young ones. Even though one might explain the turbidity by the disappearance of lecithin, a well-known colloid stabilizer, the author states that the rate of lecithin hydrolysis did not appear to be related to the time required for the development of turbidity.

Compared to the mass of data accumulated on serum lipides little effort has been spent on the study of the lipide composition and metabolism of the atheromatous aorta. Lundbaek & Petersen (122) analyzed fatty coronary arteries from diabetics and others. The cholesterol-phospholipide ratio of the artery was approximately twice that of plasma. The outstanding difference between arterial and plasma phosphatide was the large amount of sphingomyelin present in the artery. Moreover, diabetic arteries were found to contain relatively more cephalin and less lecithin than the nondiabetic ones. The metabolism of aortic phospholipide in rabbits on high cholesterol diets was studied by Zilversmit *et al.* (104) and Shore *et al.* (123). The synthesis of P^{32}-labeled plasma phospholipides in these animals was markedly increased, but, surprisingly enough, little or no deposition of plasma phosphatides in the atheromatous aorta could be detected. The accumulated phosphatide of the fatty plaque apparently resulted from a greatly increased rate of synthesis of aortic phospholipides *in situ*. It appears, therefore, unlikely that intact

plasma lipoprotein molecules contribute their lipide to the formation of atheromatous lesions. It is interesting to speculate that the accelerated synthesis of aortic phospholipide is a specific response of this tissue to remove the excess cholesterol, or is somehow connected with the "reaction" of damaged tissues in general.

Phosphatides in other diseases.—The alteration of serum lipides in disease may be the result of a specific derangement of lipide metabolism or may be brought about by the simultaneous presence of malnutrition, hemoconcentration, or nonspecific stresses such as starvation. For this reason no detailed account of the many clinical serum lipide studies will be given. However, since starvation is a common component of many diseases the studies of Petersen (124) deserve separate attention. During a seven day fast of normal adults, plasma phospholipide concentrations reached a maximum in four to five days. The increase in plasma phosphatides could be accounted for by a rise of 40 per cent in the concentration of sphingomyelin without any alteration in the lecithin or cephalin fractions. This author reports the following values for normal postabsorptively determined plasma phosphatides: lecithin 143, sphingomyelin 48, and cephalin 10 mg. per cent.

Serum phospholipides in kidney disease have been reported by Svanborg *et al.* (125) and Hartmann & Schulze (126). Kidney poisoning in rabbits has been studied by Bauer *et al.* (127). Various liver diseases have been intensively investigated by Bonelli (128), Kjerulf-Jensen & Marner (129), and by Petersen (130). Goldbloom *et al.* (131, 132) measured serum lipide concentrations in a variety of diseases. Phospholipide alterations in rheumatic fever were reported by Wallis & Viergiver (133).

Phosphatides in blood coagulation.—A strong antiheparin material with thromboplastic activity has been isolated from the water-insoluble remnants of blood platelets and was identified as a phosphatide [Van Creveld & Paulssen (134)]. The action of lipides and their hydrolysis products on clotting of fowl plasma was studied by Hecht & Ottens (135) and by Hecht (136). These investigators showed that phosphatide preparations contaminated with mere traces of cadmium chloride inhibit coagulation and thereby mask the thromboplastic effects of the lipide. Cerebrosides, lecithin, sphingomyelin, inositol-phosphatide, and all breakdown products of phosphatides were ineffective as thromboplastic agents, whereas ethanolamine and sphingosine slowed the clotting process. Apparently only cephalin effectively accelerates blood clotting, and, according to Maltaner *et al.* (137), only phosphatidylserine shows a strong thromboplastic activity. The effectiveness of soybean phosphatide as a substitute for the platelet factor in thrombocytopenic, x-ray treated dogs was investigated by White *et al.* (138). Addition, *in vitro*, of the phosphatide to thrombocytopenic blood markedly increased prothrombin utilization, but, *in vivo*, the phosphatide produced only a temporary decrease in clotting time without any change in platelet count or clot retraction. The mechanism of anticoagulant action of inositol phosphatide was studied by Rapaport *et al.* (139).

Liver Phosphatides

Phosphatide synthesis by cell components.—Kennedy (140) has studied the synthesis of phosphatides by isolated mitochondria and mitochondrial enzymes, using P^{32} and C^{14} as labeling agents. Oxidative phosphorylation was required for the incorporation of phosphate into the phosphatide molecule and α-glycerophosphate appeared to be an intermediate in this reaction. Kornberg & Pricer (141) similarly demonstrated the enzymatic esterification of α-glycerophosphate to form phosphatidic acid in the presence of ATP^2 and coenzyme A. Kennedy found that the incorporation of choline into phosphatides by mitochondrial enzymes was stimulated by the addition of ATP, phosphatidic acid, cysteine, and coenzyme A and did not involve phosphorylcholine as an intermediate. On the other hand Kornberg & Pricer (142) investigated the synthesis of phosphatides *in vitro* using a different enzyme preparation and found phosphorylcholine to be a precursor of the phosphatide molecule. Perhaps there are two different paths by which choline is incorporated into phosphatide. In livers of intact guinea pigs Dawson (143) found that glycerylphosphorylcholine and glycerylphosphorylethanolamine could not be the immediate phosphorus precursors of their respective phosphatides but could be their breakdown products.

Griffiths & Pace (144) and Lévy *et al.* (145, 146) found the rate of synthesis of phosphatides in different liver cell fractions of intact rats to be rather uniform. This contrast to the turnover of nucleic acids, which depends greatly on their location in the cell, suggested to the authors that phospholipides are structural elements of the cell (144) or do not participate preferentially in fat metabolism (145). Barnum & Halberg (147) showed that P^{32} in microsome phosphatides was higher at night than during daytime. The difference appeared to be inversely related to the eosinophil counts. Carbon tetrachloride poisoning increased the synthesis of P^{32}-labeled phosphatides by liver mitochondria [Christie & Judah (148)]. This may, of course, represent accelerated mitosis during regeneration of liver tissue, which has been shown to increase phosphatide synthesis [Johnson *et al.* (149, 150)]. Jedeikin & Weinhouse (151) have studied the incorporation of C^{14}-labeled palmitate into the lipides of rat liver slices and homogenates. The fasted liver showed a depressed phosphatide synthesis and a diminished fatty acid oxidation. The same parallelism prevailed when the concentration of fatty acid in the homogenate was increased. Phosphatide formation from fatty acid was strongly depressed by anaerobic conditions and various enzyme poisons, and enhanced by the presence of coenzyme A, suggesting that phosphatide synthesis is associated with respiratory activity.

Dietary effects on liver phosphatides.—Various aspects of liver phosphatide metabolism have been thoroughly reviewed by Artom (152). Recent developments in this field touch upon the relation of lipotropic agents and amino acids to liver phosphatide metabolism. Raman (153) found that the addition of choline to high-fat or fat-deficient diets did not alter the amount of carcass phosphatides or liver phosphatides expressed per 100 gm. of body

weight. Dogs maintained on high-fat, low-protein, choline-deficient diets for one to three weeks showed a marked lowering of the choline-containing phosphatides of liver [DiLuzio & Zilversmit (154)]. Slices obtained from these livers showed an impaired ability to synthesize phosphatides and, in contrast to normal liver slices, were stimulated by the addition, *in vitro*, of choline. Liver slices, homogenates, or washed particles from rats on choline-deficient diets did not oxidize C^{14}-labeled palmitate or stearate as rapidly as similar preparations from normal liver [Artom (155)]. Administration of choline, orally or parenterally, increased the fat oxidation *in vitro*, but the addition, *in vitro*, of choline was without effect. These findings support the hypothesis that the lipotropic activity of choline is mediated through increased fat oxidation.

Cornatzer & Gallo (156) concluded that the rate of phosphatide synthesis in liver, relative to that of the nucleoproteins, is not affected by changes in protein or fat intake, by the histological appearance of the liver, or by supplementation of the diet with chlortetracycline or sulfasuxidine. The results are not easily evaluated, since they are given as "relative specific activities per gm. of fat-free tissue." Horst & Tepe (157) confirmed that the administration of choline to normal human beings does not alter the rate of appearance of radioactive serum phosphatides. Hartmann *et al.* (158, 159) studied the action of lipotropic agents on liver phosphatides of dogs poisoned with carbon tetrachloride. The lowered plasma and liver phosphatides were rapidly restored by the administration of choline, methionine, or inositol. Both methionine and inositol increased the rate of appearance of P^{32}-labeled plasma phospholipides. The authors suggest that the excess liver fat is converted to phosphatide during the recovery phase. Schettler (160) also observed low concentrations of phosphatide in liver and plasma in dogs poisoned with carbon tetrachloride and in patients with fatal liver disease. In contrast to its action in dogs, carbon tetrachloride increased the liver phospholipides and triglycerides of rats [Hartmann *et al.* (161)]. The triglycerides were returned to normal most effectively by a combination of methionine and fructose, a preparation that had no effect on the high liver phosphatide levels. After damaging rat livers with carbon tetrachloride or removing part of the liver surgically, acetal-phosphatide concentrations in the regenerating liver rose to very high levels [Yarbro & Anderson (162)]. Hartmann & Fleck (163) compared the chemical and histological analysis of fatty livers. They discovered a good correlation between the triglyceride-phosphatide ratio of a liver sample and the amount of histologically demonstrable lipide. When the liver phosphatide decreased more rapidly than the triglyceride concentration, an increase of histologically demonstrable fat was reported. The authors suggest that in the liver, as has been postulated for plasma, the phosphatides prevent the coalescence of submicroscopic fat droplets.

The effect of dietary cholesterol on liver phosphatide metabolism has been reported by Clément *et al.* (164, 165, 166). These investigators ob-

served a 25 per cent decrease in liver phosphatides when rats were maintained on a high cholesterol diet for 6 to 10 months. However, per gm. of liver protein, the phosphatide concentration remained unchanged. A definite decrease in liver phosphatide turnover was observed even a month after removal of the cholesterol. Apparently liver phosphatide metabolism differs in the rabbit, since this species showed some increase of liver phosphatide concentration and turnover while on a high cholesterol diet [Zilversmit *et al.* (104)]. Clément & May (167) studied liver lipide metabolism with the aid of conjugated fatty acids. Eleostearic acid fed as free fatty acid or triglyceride was found in the liver phosphatides of rats, but the tetraethylenic C_{18}-acid, parinaric acid, did not participate in phosphatide synthesis even though it was rapidly incorporated in liver triglycerides. Apparently the metabolic destiny of fat depends on its degree of unsaturation.

The effect of x-radiation on liver phosphatides cannot yet be ascertained. Coniglio *et al.* (168) reported an increase while Davison *et al.* (169) found a decrease in these lipides. Both studies recognized that decreased food intake played a part in the lipide changes, a fact well illustrated by the studies of Wikramanayake *et al.* (170, 171). These authors showed that an increased caloric intake increased the quantity and turnover of liver phosphatides only when adequate amounts of protein were present in the diet, indicating that a deficiency of protein may limit phosphatide synthesis by a lack of factors other than choline. This interpretation is compatible with the observation that choline did not restore liver phosphatide levels or liver function in dogs or rats on a choline-deficient low-protein diet [DiLuzio & Zilversmit (154); Artom & Swanson (172); Koch-Weser *et al.* (173)]. The studies of Harper *et al.* (174) and Singal *et al.* (175) indicate that amino acids such as threonine may be the limiting factor in these diets. Singal *et al.* (176) demonstrated that the liver phosphatide concentration and turnover is decreased in threonine-deficient rats and that the administration of threonine at the time of P^{32} injection returned the phosphatide turnover to near normal levels. Interestingly enough the small intestine and kidney of the deficient animals did not show a depressed phosphatide metabolism.

Hormonal control of liver phosphatide metabolism.—Among the many difficulties encountered in the interpretation of experiments on the hormonal regulation of tissue metabolism is the well known fact that the removal or stimulation of one gland results not only in some direct action on a target organ but ordinarily also affects the function of other endocrine glands. Moreover, two hormones produced by the same gland, e.g., the adrenals, may antagonize each other to some extent. It is thus not surprising that certain experimental inconsistencies have made their appearance in the literature of this field. Thus, Goebel *et al.* (177) could not find any difference in the specific activity of liver or kidney phospholipide of normal and adrenalectomized rats, but Halberg *et al.* (178) reported a definite decrease in the relative specific activity of liver phospholipide of adrenalectomized mice. Zilversmit *et al.* (73) observed that phosphatide turnover of liver, kidney, lung, small

intestine, and muscle in DCA- or cortisone-treated adrenalectomized dogs did not differ from normal. However, the exchange between plasma and liver phosphatides was markedly depressed in the DCA-maintained adrenalectomized animals and returned to normal by cortisone. Dury & Treadwell (179) found low liver phosphatide levels in adrenalectomized and in adrenalectomized-alloxanized rats with compensatory increases in neutral fat. These investigators showed, moreover, that liver phospholipide levels may be altered rapidly in response to epinephrine, insulin, or glucose [Treadwell & Dury (180)]. Aujard (181) found no change in the amount of liver phospholipides of rats injected with noradrenalin or exposed to cold, but the nonphospholipide fatty acids increased markedly. Twenty-four hours after alloxan, liver phosphatides and triglycerides appeared to be elevated [Dury (182)].

The role of the pancreas in the regulation of phosphatide metabolism was studied by Zilversmit & DiLuzio (102) who showed that pancreatectomy results in a marked stimulation of the phospholipide turnover of liver and plasma. Essentially the same findings were reported in phlorizinized dogs [DiLuzio & Zilversmit (103)]. In both these preparations the oxidation of fat was greatly increased, and the findings are compatible with the hypothesis that phosphatides play some part in fat oxidation. The early work supporting this hypothesis is reviewed by these authors. The subsequent demonstration that choline increases fat oxidation (155) as well as phospholipide formation (154, 172) and that phosphatide synthesis is coenzyme-A dependent (140, 141, 151, 183) may provide further evidence for a possible role of phosphatides in fat oxidation. On the other hand, the demonstration that choline added to choline-deficient liver slices *in vitro* stimulated phosphatide synthesis (172) but failed to accelerate fat oxidation (155) indicates that the intensity of fat oxidation is not the only rate-determining step in the process of phosphatide synthesis.

The effect of the thyroid on phosphatide turnover was studied by Venkataraman & Friedell (184) and by Cornatzer *et al.* (185). The former observed a 25 per cent increase in the relative specific activity of liver phosphatide of hyperthyroid rats, whereas the latter found that thiourea decreased phosphatide synthesis relative to the synthesis of nucleoprotein. Cornatzer *et al.* also studied the effects of pituitary hormones and found that the fat-mobilizing fraction of the pituitary increases liver lipides without increasing the rate of liver phosphatide synthesis. Growth hormone, on the other hand, was found to increase the phosphatide turnover in liver.

Borell *et al.* (186, 187) measured the effect of chorionic gonadotropin and ovarian hormones on the turnover of liver phosphatides. Gonadectomy, with or without chorionic gonadotropin, did not appear to affect phosphatide specific activities of liver or kidney. However, examination of the data reveals that the specific activity of kidney inorganic phosphate was markedly reduced in the gonadectomized rats. Consequently, conclusions about turnover rate based on the specific activity of phosphatides, rather than their relative

specific activities, must be suspect. The authors' conclusion that in hypophysectomized rats, kidney phosphatides turn over more rapidly than liver phosphatides may also be erroneous, since no account was taken of the fact that the specific activity of inorganic phosphate in kidney exceeded that of liver.

Phosphatides in Other Tissues

Brain phosphatides.—The chemistry, metabolism, and physiological significance of phosphatides in the central nervous system have been competently reviewed by Sloane-Stanley (188) and Dawson (189). After intracisternal injection of P^{32} the incorporation of this isotope into brain phosphatides of cats was found to be more rapid than after intravenous injections [Strickland (190)]. Under these conditions diphosphoinositide reached a higher specific activity than the other brain phosphatides. This was also the case in guinea pig brain slices and homogenates [Dawson (191, 192); Norman & Dawson (193)]. High specific activities in cephalin as compared to sphingomyelin were also observed by Streicher & Gerard (194) *in vitro*, although *in vivo* the specific activities did not differ so much. A similar difference between behavior *in vitro* and *in vivo* was noticed by Dawson (192) who observed that phosphatidylserine and phosphatidylethanolamine were scarcely renewed *in vitro* whereas *in vivo* they were rapidly synthesized. The multitude of factors affecting the incorporation of P^{32} into brain slices and homogenates have been investigated by Dawson (195) and by Strickland (196). The latter found, in contrast to Streicher & Gerard (194), that phosphatide specific activities of cat brain slices steadily increased during the 4 hr. incubation period. Both Strickland and Dawson found a dependence of phosphatide synthesis on the availability of energy producing substrates and oxidative phosphorylation. The addition of choline, betaine, ethanolamine, serine, inositol, glycerol, or glycerophosphate did not affect phosphatide synthesis [Dawson (195)]. An investigation of the effects of Ca^{++}, K^{+}, NH_4^{+}, and certain substrates on the incorporation of P^{32} into cat brain slices confirmed the essential role of high energy phosphates in phosphatide synthesis [Rossiter & Findlay (197)].

The effect of pituitary hormones on brain phosphatide metabolism of mice was studied by Torda & Wolff (198) and Torda (199). A single dose of adrenocorticotropic hormone appeared to increase the brain phosphatide concentration as well as synthesis from P^{32}, but prolonged administration of this hormone was without effect. Administration of thyrotropic hormone decreased the brain phosphatide content, but other pituitary, gonadal, or adrenal hormones had no apparent effects. Torda (199) also reported the effect of convulsants on brain phosphatides. Convulsions lasting 1 to 5 min. resulted in about a 20 per cent decrease of radioactive phosphatide of brain, suggesting a rapid breakdown of phosphatides. Electrical stimulation of brain mitochondria decreased the phosphatide specific activity though not as much as the specific activity of other organic phosphates [Abood (200)]. Hokin & Hokin have shown that acetylcholine markedly increased the in-

corporation of P^{32} into phosphatides of brain- and pancreas-slices (201, 202) but did not affect the incorporation of labeled glycerol into these lipides. Thus it appears that acetylcholine stimulates the turnover of only a part of the phosphatide molecule. Moldave (203) reported an increased incorporation of P^{32}, *in vitro*, in phosphatides of all cellular fractions of brain cultures containing Theiler's virus GD VII.

Tumor phosphatides.—Haven *et al.* (204) have shown that the Walker carcinoma-256 has a higher phospholipide content than the tissues of the host. In particular the alkali-resistant phospholipide fraction, presumably acetal phosphatide, was found to be elevated. Boyd *et al.* (205, 206) demonstrated not only an increased amount of phospholipide in the tumor, but also a shift towards higher phospholipide levels in muscle, small intestine, and testicles of these animals. Stewart & Begg (207) showed that force-feeding of high fat diets to rats with Walker carcinoma resulted in a tremendous increase of all plasma lipides. This suggests the inability of these animals to control lipide metabolism within normal bounds. In mammary carcinomas phosphatides were found to be relatively more abundant since other lipides had decreased; the proportion of lecithin, however, was decreased whereas cephalin was much higher than in the resting or pregnancy-stimulated gland [Johnson & Dutch (208)]. Even though the phosphatide content of rabbit papilloma was below that of skin, the synthesis of these lipides was significantly greater in the tumor tissue [Cornatzer *et al.* (209)]. The incorporation of P^{32}, *in vitro*, into normal and leukemic mouse tissues has been studied by Williams *et al.* (210), who found a folic acid antagonist to be without effect on the synthesis of liver or tumor phosphatides. The phospholipide content of whole human leucocytes was found to be unaffected by leukemia, but the nuclei showed a 50 per cent decrease in phosphatide content [Polli & Ratti (211)].

Other tissues.—Phosphorylations in hog-kidney nuclei apparently do not depend on high-energy phosphates since various enzyme poisons were shown not to interfere with phosphatide synthesis from P^{32} *in vitro* [Lang *et al.* (212); Siebert *et al.* (213)].

Rat skin has been shown to be one of the few tissues that cannot synthesize its own phosphatides *in vitro* [Leonhardi *et al.* (4)]. Experiments with partially hepatectomized rats, fed labeled phosphatide, indicated that skin normally obtains its phosphatides from plasma (4). The finding that severely burned rat skin contained less radioactive phospholipide after P^{32} administration [Lee *et al.* (214)] may therefore reflect a decreased uptake of phospholipides rather than a diminished synthesis.

When a P^{32}-labeled phosphatide emulsion was administered intravenously to rats, the amount of labeled depot-fat phosphatide was much less than when P^{32} was given as phosphate [Chevallier & Schneider (215)]. These authors concluded that subcutaneous fat synthesizes its own phosphatides. However, in the dog an appreciable portion of the depot phosphatides appears to be derived from plasma [Harper *et al.* (100)]. Chevallier *et al.* (216)

noted that the phosphatides of perirenal fat turn over more rapidly than those in subcutaneous fat.

Factors influencing the incorporation of P^{32} into pigeon pancreas slices were studied by Hokin & Hokin (217). In the early work a parallelism between phosphatide turnover and enzyme secretion was noted, but later studies (202) indicated that the parallelism ceased at higher concentrations of acetyl choline or when eserine or choline were added to the slices. Moreover, the incorporation of P^{32}, *in vivo*, into the pancreata of mice did not appear to depend on the secretory activity of this gland (202).

Thyrotropic hormone has been shown to stimulate phospholipide synthesis of beef thyroid slices without affecting this process in liver or kidney slices [Morton & Schwartz (218)]. Adrenocorticotropic hormone similarly restored the diminished phospholipide synthesis in the adrenal of hypophysectomized rats [Riedel *et al.* (219)].

The lipides of heart muscle were studied by Schulze & Südhof (220). Hypertrophy of this organ by exercise (guinea pigs) or occlusion of the aorta (dogs) was associated with an increased phosphatide concentration. When cardiac insufficiency was approached the phosphatides decreased. During these alterations in the heart the adrenal phosphatides remained constant [Schulze (221)].

Phospholipides of bile have been investigated by Polonovski & Bourrillon (222). These investigators found only one phosphatide, namely lecithin, and could not detect free choline when degradation of lecithin was prevented. They emphasized the special procedures required for the extraction of bile phosphatides. Essentially the same findings were reported by Etienne-Petitfils & Kahane (223). The absence of cephalin and sphingomyelin from human bile was also observed by Isaksson (224) who studied the emulsifying power of lecithin-bile salt complexes present in bile (225).

Conclusion

During the past few years the metabolism of the complex lipides has been studied intensively by biochemists, physiologists, and clinical investigators. The student with isolated enzymes, cell fragments, tissue slices, perfused organs, and intact animals has suffered from the relative inadequacy of methods for the separation and estimation of lipides in small samples of blood and tissue. It would appear that the intense application of existing techniques and the development of new ones would be of inestimable value to the students of lipide metabolism who are probing for greater comprehension of the metabolic pool and those who are attempting to use the lipides as diagnostic tools in the clinical laboratory.

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CHEMISTRY OF PROTEINS, PEPTIDES, AND AMINO ACIDS[1]

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Introduction.—In such a review as this, whose subject matter includes fields as widely different as organic chemistry and the mathematics of physical chemistry, the practicable falls far short of the ideal. Ideally the reader might hope for a comprehensive guide to the year's literature, critical and constructive comment throughout its range, and perhaps also a general assessment of the position which knowledge within it has reached and a prospect of future developments. Such a task is impossible for a single author, suffering his own limitations as well as those of time and space.

Recognising these limitations, and the dryness and inconvenience of a text which merely mentions published work without adequate comment, the reviewer has attempted a separation of bibliography from comment.

The more useful part of the review is a classified list of references. These have been given under broad subject headings with some attempt at arrangement within each subject; a few cross-references are given. Each paper has a brief descriptive title (usually not the author's title). This arrangement should help a reader to find quickly what work has been done within any field, and to form his own opinions about it by reference to the original works. The bibliography is moderately comprehensive of works in English, French, and German; publications in other languages have not been included—the reviewer cannot read them and there has been no time to obtain translations. He makes apology to the authors of works omitted because of the curse of Babel, and to others whose works have not been included through inadvertence. Papers on x-ray analysis and its interpretation, and those on polymer chemistry not distinctly within the protein field, have not been systematically quoted; only those are referred to which happen to have come to the reviewer's attention.

Hoping that he has in this way discharged one duty to the reader, the reviewer has felt free not to attempt comprehensive comment. The rest of the review, therefore, consists of a short essay in which he has allowed his ignorance and prejudices full rein, and in which direct reference is made only to a small number of papers.

One is first impressed by the sheer bulk of published work. One wonders next whether much of this will not be lost from notice in the general spate of publication, and then whether this fate may not often be deserved. Has enough consideration always been given to the likely significance of the results of any particular piece of work, before it was undertaken and during

[1] The survey of the literature pertaining to this review was completed on September 30, 1954.

its performance? Although it must be assumed that all correct observations can be fitted sooner or later into the general scheme of knowledge, human frailty makes it likely that much work will be forgotten before it has become important. Biochemical researchers might, perhaps, ask themselves from time to time whether their work is directed, however obliquely, towards the main biochemical objective. Within this field the objective is to discover what amino acids and their polymers are doing in biological systems, how they got there, and how their presence, formation, and "functions" are to be accounted for in chemical terms. This is not to say that work undertaken with other objects in view is not legitimate; for example, a polymer chemist may find a protein to be an interesting object of study, ready to hand; but although his results might one day prove to be useful to the biologist, such work is not to be regarded, at the time of doing, as Biochemistry.

The best hopes of achieving the biochemical objective seem to lie within the following broad fields of work: (*a*) exact definition of the objects of study in a chemical sense, that is, the specification of the purity and homogeneity of substances which are regarded as biochemical entities; (*b*) definition of their compositions and structures, in the sense of organic chemistry; (*c*) definition of their stereochemical configurations; (*d*) definition of their physico-chemical relationships with other substances with which they come into contact in biological systems; (*e*) correlation of any or all of these with their biological activities. Work is being actively carried out in all these fields.

Purity or homogeneity.—The separation of proteins and polypeptides from the complex systems in which they occur has always been a matter of interest. Following on the widespread achievement of the crystallisation of proteins, and the application of the ultracentrifuge, both begun late in the 1920's, it has been a general article of faith that proteins can be obtained in a chemically pure state; that is, that each particle of a given (biologically defined) protein, is exactly like every other particle. Whether or not this hypothesis should prove to be true, it is certain that evidences of the homogeneity of preparations, especially that of crystallinity, have been too uncritically accepted.

In the last few years a considerable change of opinion has been seen. The development of improved methods of testing homogeneity, especially by boundary analysis in electrophoresis and sedimentation, by ion-exchange chromatography, by countercurrent distribution, and by variable-solvent solubility has led to a healthy scepticism about earlier conclusions. One finds Taylor and Edsall (in 353), Craig (in 3), and others sporadically, giving careful discussion to questions of how homogeneity is to be achieved and tested. On the whole, the reviewer thinks that most views expressed are still too optimistic. An excellent review by Colvin, Smith & Cook (3a) should be read by all workers in this field. They survey all the main methods of testing homogeneity and arrive at the conclusion that the present evidence does not justify the acceptance of any preparation yet achieved as strictly homogeneous,

with the possible exception of insulin separated by countercurrent distribution.

The crystallisation of a preparation is obviously still regarded by many as at least a "rest and be thankful." Smithies' (75) work on β-lactoglobulin is of special interest here; he shows that the components of this protein show no sign of changing their proportions on repeated recrystallisation; only when conditions are carefully chosen so that the solid phase is not crystalline can fractionation by salting-out be achieved. This conclusion may apply elsewhere, and the application of the variable solvent solubility test over a range of conditions should find increasing use.

The question of homogeneity may have two kinds of importance. First, separation and the testing of homogeneity usually precedes other work on the material, including its analytical investigation, study of its physical properties and of the dimensions of its particles; the question should always arise whether the material available is sufficiently homogeneous to make further investigation worth-while. The answer to this question depends so much on the nature of the heterogeneity and on that of the further investigation, that no rules can be laid down; the question must be considered afresh in each particular instance. One certainly cannot label all work done on heterogeneous preparations as invalid. For example, the main chain structure of insulin has certainly been established, even though the possibility remains that a minor proportion of its molecules may differ in composition from the majority; the same conclusion probably applies to much of the work done on end-group and sequence assays. Many molecular weights, and other physical parameters can also be taken as, at worst, average values dominated by a majority group of identical, or very closely similar, particles.

Secondly, the idea seems to be gaining ground that all the particles of a biologically-defined material may not be of absolutely uniform composition and configuration. The demonstration that insulins of different species differ in their composition (188) might support such a thesis; on the other hand, oxytocin of undoubted chemical purity has now been completely synthesised and is fully active (320). Evidence at present available cannot decide this question, and it will not be easy to decide. Heterogeneity of a separated protein might result from an intrinsic heterogeneity of the original, biologically defined material, or it might be the result merely of handling or of insufficient removal of contaminants; conversely, if the native material is intrinsically heterogeneous it should always be possible to make very uniform fractions from it by a sufficient application of fractionation procedures. The solution of this problem is of some importance to ideas of protein and peptide biosynthesis.

In either of these contexts, the resolving power of the method of testing heterogeneity has to be borne in mind. For example, the fact that countercurrent distribution resolves two forms of insulin differing only with respect to a single amide group may mean very little; electrophoretic boundary-spreading is capable of similar sensitivity. Solubility-analysis and sedimenta-

tion analysis reveal different, in the latter case perhaps more gross, types of heterogeneity.

Finally, some care may be needed in defining and interpreting heterogeneity, particularly that revealed by dynamic methods. Evidence of mobile association-dissociation processes, occurring between particles of the same or of different proteins, continues to accumulate. Such behaviour can both lead to the appearance of heterogeneity in systems which are chemically homogeneous and obscure the appearance of heterogeneity in systems containing more than one chemical component.

Methods of observing heterogeneity.—Among transport methods, paper electrophoresis and electrophoresis in other supporting media, continue to be refined and made more quantitative, and the two-dimensional versions of this technique will prove of great value in separating proteins. Countercurrent distribution, perhaps the most powerful of these methods, is still not applicable to most proteins, but the search for suitable immiscible solvent mixtures continues. Various types of adsorption, ion-exchange, and phase-partition chromatography have been described.

Williams (95) has given a useful review of the uses of dynamic methods, including the combined use of sedimentation equilibrium and velocity, for the quantitative description of heterogeneity. Baldwin (96) has improved and simplified methods of sedimentation boundary analysis and has shown how the distribution of the sedimentation coefficient can be obtained. Two further papers of this series, one applying to the boundary analysis of nearly-homogeneous material, are due to appear shortly.

Akeley & Gosting (105) have made an exact analysis of the distribution of Gouy fringes in the diffusion of two solute components. Unfortunately, this has not led to a method of working back from the Gouy fringes to the composition of an unknown solute mixture. Their work, therefore, represents a considerable theoretical, but not so great a practical, advance upon earlier analysis (345). Charlwood (346) and Herdan (106) have shown how the moments of the schlieren boundary curve can be used to assess diffusion heterogeneity. In spite of these researches, diffusion remains a method of low resolving power; the distribution of molecular weight, in a heterogeneous mixture, can still not be obtained except by use of the equilibrium ultracentrifuge. With a new type of instrument becoming available, this technique may come into wider use.

Molecular dimensions.—With the increasing availability of instruments, it is becoming frequent for measurements of the sedimentation coefficients, often also of diffusion coefficients, of partial specific volumes and sometimes of the intrinsic viscosities of protein preparations to be quoted. Most of these measurements are probably reasonably precise. Sometimes measurements have been made over a range and extrapolated to zero concentration.

The reliability of measured sedimentation coefficients has been in some doubt in the past. Recent experience has shown a fairly close agreement between values obtained in carefully calibrated oil-turbine instruments and in

Spinco electrically driven instruments. A doubt has remained with regard to the temperature in the Spinco rotor. Biancheria & Kegeles (91) have confirmed, by melting point measurement, Waugh & Yphantis' finding (344) (by use of a radiation thermocouple) that the Spinco rotor temperature falls by 0.9°C. between rest and 1000 r.p.s. This means that directly observed sedimentation coefficients measured at near 20°C. have to be increased by 2.2 per cent, and eliminates the remaining discrepancy between the two types of instrument.

Baldwin & Ogston (100) have shown that, at least in a binary system, the anhydrous molecular weight is obtained from sedimentation and diffusion coefficients extrapolated to zero concentration, and that solvation of any type has no influence on the result. Haase (101) has obtained the same result, by the method of irreversible thermodynamics. Unlike Baldwin & Ogston, he states that no assumption is required about the similarity of the microscopic dynamic processes in sedimentation and diffusion in order to arrive at this result. On common sense grounds it seems difficult to accept this conclusion, and one wonders whether some tacit assumption may not be hidden in Haase's derivation. Most actual systems are ternary or higher, and it has to be remembered that the assumption, that the solvent (water plus salts etc.) mixture is behaving as a single component, may not be strictly justified. Lansing & Kraemer (350) showed that the treatment of multicomponent systems as though they were two-component might lead to small errors in estimates of molecular weights, even using values of sedimentation and diffusion coefficients extrapolated to zero concentration of solute.

The usefulness of molecular weight determinations, on reasonably homogeneous material, is not to be doubted. A physically determined molecular weight is useful in conjunction with various sorts of analytical data. Kilby & Youatt (347), for example, have shown that there is fairly good agreement between the stoicheiometric units of trypsin and chymotrypsin which react with phosphoric ester inhibitors and the molecular weights. The significance of the molecular weight may, however, be doubtful where association-dissociation occurs, as the history of insulin has shown. Parrish & Mommaerts (129) have shown that the sedimentation coefficient of myosin is a function of the temperature. In such cases the value may be a dynamic mean and may apply only in a limited range of conditions. Steiner (99) has discussed the derivation of the properties of the constituent particles of such a system from equilibrium and dynamic measurements.

The deduction of other molecular parameters from dynamic measurements is a much more hazardous business, and the results are often of doubtful value. The popular quantities to calculate are "shape" and "hydration." Ogston (93) has emphasised that calculation of these quantities relies, not only on hydrodynamic theories, but fundamentally on the choice of a model to which to apply those theories. The choice of a model, which to a large extent predetermines the results, is in turn determined by their theoretical tractability and is at present limited to three: the spheroid, rod, and coil.

Even where the validity of the hydrodynamic theories themselves is not in doubt, serious discrepancies can arise. Sadron (351), for example, finds values for the axial ratio (or ellipticity) of the spheroidal model of serum albumin from 3.3 to 7.9; Tanford & Buzzell (124) find a value for Scheraga & Mandelkern's β for serum albumin which is below the theoretical minimum value. Schulman (128) has reviewed various hydrodynamic data for fibrinogen and finds that the quantities deduced vary considerably with the data used to obtain them. Evidently such measurements of shape cannot be relied on to give more than a rough quantitative indication. For such a use, the variation of sedimentation coefficient with concentration seems to be reasonably satisfactory. Wales & van Holde (94), using the expression for the frictional coefficient of a random coil instead of that for a spheroidal particle, have obtained equations closely similar in form to Ogston's (93).

The problem of measuring "hydration" is difficult because hydration and shape combine to affect dynamic properties, and therefore their values are interdependent and both depend on the choice of model. In addition to this difficulty is that of defining hydration, to which Edsall (in 2) has drawn attention. Any definition must, of course, be related to some method of measurement if it is to mean anything. The trouble is that definitions, and the resulting values, based upon different methods of measurement, do not agree. This disagreement is not surprising when one considers the resemblance between the amount of a hydrate shell and the thickness of an interphase.

The following examples illustrate how various methods of measurement lead to different, though possibly related, quantities. (*a*) Measurement of the distribution of water and salts in crystals and of the vapour pressure of water over moist crystals (154, 155) reveal the variations of thermodynamic quantities with composition; these may indicate differences between "chemical" and "physical" hydration, though it is dangerous to press this distinction too far. (*b*) Hydrodynamic methods usually measure the amount of solvent which moves with the solute particle in excess of what would be expected on the basis of some particular model; this amount obviously depends on the model chosen. (*c*) The method of finding the density of a solution in which the rate of sedimentation is zero, exemplified by the work of Sharp & Beard (141), measures a "selective solvation," analogous to the Gibbs surface excess of an interphase.[3]

Electron microscopy seems likely to give unambiguous information about the shape of dried particles; this may differ from the shape in solution, and obviously no light is thrown on hydration. Schmidt, Kaesberg & Beeman (153) have made a striking investigation of the turnip yellow virus, and its nucleic acid-free protein, by low angle x-ray scattering; the results indicate a water-filled protein shell, in good agreement with other evidence. Light-

[3] The demonstration of this will be given by the reviewer elsewhere [*Trans. Faraday Soc.* (In press)].

scattering perhaps gives the best indications of shape in solution, but is also dependent for its interpretation in this sense on comparison of results with those predicted theoretically for certain models.

In view of the difficulties of interpreting measurements of "shape" and "hydration," one wonders whether efforts to this end may not often be misdirected, except in those cases where a clear qualitative answer can be hoped for, e.g., as to whether a given type of particle is compact, stiff and greatly elongated, or a flexible coil. For example, Harkness & Wasserman (130) conclude that actomyosin has a variable flexible-coil structure, agreeing with Ogston's conclusions (93); it seems clear, in this case, that the molecule has not the usual compact form, but is not a rigid rod.

Chemical structure.—The year has seen a large number of papers on the amino acid analysis of proteins, on the structure of peptides obtained from them by partial hydrolysis, and on the partial structure (end-group or end-sequence assay) of intact proteins and peptides. The established methods of end-group and sequence assay seem to give reasonably clear cut results. An encouraging amount of effort has been given to their modification and improvement. Work of this kind is of vital importance in supplying a chemical background to our understanding of protein structure. Its initial success with insulin has been followed by the complete definition of the structures of oxytocin and vasopressin, and by the synthesis of the former (320). In a different direction, interesting light has been thrown on the changes involved in the activation of trypsinogen and chymotrypsinogen. Both these activations involve peptide-splitting. It is interesting that the liberation of a particular end-group may not be essential to activity, since an active chymotrypsin, without alanine end-group, has been prepared (10).

Complete amino acid analysis, with a good precision, now seems to be regularly obtained by varying chromatographic procedures. Analysis, though useful as a check, is clearly much less informative than series assays. In view of the laboriousness of series assay procedures, it is not clear that the objects of study are always well chosen. Any considerable heterogeneity makes the results difficult to interpret; in this respect serum albumin (whose homogeneity is suspect) seems to be a poor choice; its complexity would also make complete structural analysis a long job, and its lack of marked specific biological properties might make the result, when obtained, uninteresting.

Analysis and series assay can themselves throw light on homogeneity. To prove homogeneity by these means it is necessary to show exact stoicheiometric relationships of all the radicles concerned and to account fully for all the starting material. Naturally, the possibility of this is limited by experimental accuracy, and proof can never be complete. Complete homogeneity of the oxytocic polypeptide has probably been proved. One cannot be quite so sure about insulin. Sanger's achievement with insulin is nevertheless very great. In locating the disulphide bridges (148), he and his colleagues seem now to have obtained all the information about a single protein which these kinds of method can hope to yield.

Configuration.—The almost universal loss of specific biological properties of proteins on denaturation shows that chemical analysis alone cannot explain these properties, and that there must be important configurational conditions to be fulfilled. The most helpful and direct attack on this problem seems to be the combined use of x-ray scattering and model structures. The systematic investigation of possible models by Pauling's school continues actively. The chief deficiency of such work, at the present, seems to be in not making allowance for side chains. This omission may not be important; Koltoun, Waugh & Bear (151) have found that insulins which have undergone side-chain modification give very similar x-ray patterns. When it comes to more specific model-making, the inexpert is left bewildered. Three spiral structures have recently been proposed for collagen (161, 162, 348), each claiming to satisfy all the known facts; one of them (162) has come in for severe criticism (163, 164).

The study of denaturation is the other main route of attack. By its nature, this kind of investigation cannot give the same detailed information as structural and x-ray studies. Yet it has led to a generally consistent and satisfactory view of the way in which the native structure is maintained and one which is, on the whole, consistent with the Pauling postulate of maximum hydrogen bonding. Three studies by Linderstrøm-Lang and his collaborators on insulin illustrate the value of this approach. They have shown, first (144), that insulin is dissociated into units of molecular weight 6000 in concentrated solutions of guanidine hydrochloride; this indicates the involvement of hydrogen bonding in maintaining large structures. Secondly (150), they have confirmed this by showing that a number of hydrogen atoms in native insulin, approximately equal to the number of peptide bonds, exchange only very slowly with D_2O. Thirdly (145), they find that the separated A chain of insulin has an optical rotation characteristic of denatured protein, in contrast with that shown by native insulin. These all support the hypothesis that in the native protein the chains are in a characteristic steric configuration and are stabilised by hydrogen bonding (147). Cecil (349) has shown that even so small a peptide as glutathione shows changes of disulphide reactivity in concentrated urea solution ascribable to the breaking of intra-molecular hydrogen bonds; this may be regarded as an analogue of denaturation.

The exhaustive study by Kauzmann and his colleagues (169 to 174) of reversible denaturation has led to the picture of one or more peptide chains held in specific configurations by bonds of varying strength; many of these are believed to be hydrogen bonds (particularly the weaker), while the stronger may include bonds such as disulphide. Denaturation involves, initially, the breaking of a number of these bonds, thus giving to the molecule a looser and more random structure, while leaving it still held in some degree of organisation by stronger bonds. At this stage, reversibility is possible because the probability of regaining the original configuration is not too small. Complete restoration of the original structure may not be necessary for the recovery of biological properties; Kleczkowski (181) has shown that TMV

(tobacco mosaic virus) and chymotrypsin are initially made more liable to thermal inactivation by ultraviolet irradiation, but not actually inactivated. More extensive disorganisation involves further bond-breakage, and is irreversible. This general view is supported by observations such as those of Yang & Foster (176) and Gutfreund & Sturtevant (176a) on the effect of acid in producing an initial "swelling" of the serum albumin molecule.

Denaturing agents are either small molecules such as H^+ (178, 182), formate ion (177), urea and guanidinium which can be expected to interfere with hydrogen bonding, or relatively large molecules, such as those of detergents, whose affinity for charged or other groups leads to their "elbowing their way in." The apparently "all-or-none" character of the denaturation observed by Yang & Foster (267) shows that a limited disturbance of this kind may unstabilise the whole structure.

The limitation, in at least some proteins, of the "active centre" to a small part of the structure raises the question of what all the rest is for, and particularly why it should have a (more or less) specific composition, configuration, and size. On the template hypothesis, this question might be answered by saying that the nature of the synthetic mechanism determines the particular structure for each protein, though a different structure might conceivably be substituted for all but the active centre without altering the activity. An alternative view might be that, once the structure of the active centre has been determined (by some limited template mechanism) a condition of maximum stability is thereby set up for subsequent stages of synthesis; and that this continues, each stage of synthesis determining the structure of maximum stability for the ensuing stage, until the structure closes upon itself and no further addition becomes possible. This idea, though highly speculative, implies certain possible consequences capable of experimental or theoretical test: (*a*) that the stability of the structure should depend fairly strongly upon the nature and arrangement of the side chains; (*b*) that such a dependence might not be absolute and that a certain degree of variation within the structure of a given active protein might be expected. The differences of composition of different insulins already mentioned (188) would be consistent with such a view though, since the differences are interspecies ones, they would be equally consistent with the full template hypothesis. The specific biological activity of so small a peptide as oxytocin, in which there seems to be no strict configurational requirement, raises the question whether the presence of a large nonactive mass in most proteins is merely accidental: if so it should be possible to synthesise quite small peptides with enzymatic activity. In this connection, Binkley (352) has given a useful summary of the evidence that enzymatic activity may reside in the nonpeptide structure of nucleic acid.

Protein and peptide interactions.—There have been many reports of work on protein-protein and protein-peptide interactions, and of proteins and peptides with smaller molecules. In general, since proteins occur naturally in systems containing a large variety of other substances, the understanding of

such interactions must be of great importance to our understanding of organised biological systems. Most of the systematic work towards this understanding is in the hands of enzymologists and is not reviewed here. Other investigations are bewildering in their variety. Often they can be seen to have some reasonable limited objective, such as the understanding of how given types of biochemical substance (such as steroids) are carried in the blood, or of the effect of artificial agents (such as detergents) on protein stability. Sometimes their objective is not obvious.

Miscellaneous.—Attention is drawn to the following technical matters not already mentioned: Baldwin's method (92) and the use by Brown and others of the Archibald method (92a) for studying the sedimentation in the ultracentrifuge of material of relatively small molecular weight; the generalised treatment by Trautman and others (98) of two sedimenting components, with the important demonstration that under certain conditions convexion may arise in such a system; Schachman & Harrington's (102) discussion of the uses of the "synthetic boundary" cell; Backus & Williams' (104) elegant method of measuring sedimentation rates in a preparative ultracentrifuge by use of floating, "self-aligning" capsules; Trautman & Burns' (118) use of a phase-plate in schlieren optical systems; Smithies' (113) extremely accurate dynamic osmometer and ingenious method of making small collodion thimbles; the use of Fe^{+++} by Block and others (66, 67), for fractionating proteins; Dervichian & Mossé's (68) beautiful and exhaustive work on the phase-diagrams of protein-salt-water systems; Katz's (73, 74) finding that hydrogen fluoride and trifluoroacetic acid can be used as solvents for proteins.

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[3] The following abbreviations are used: ATP for adenosinetriphosphate; D for diffusion coefficient; DNB for dinitrobenzene; DNP for dinitrophenol; DPNH for diphosphopyridine nucleotide (reduced form); EM for electron microscopy; FDNB for fluorodinitrobenzene; M for molecular weight; OP for osmotic pressure measurement; S for sedimentation coefficient; SH for sulfhydryl (or sulfhydryl compounds) TMV for tobacco mosaic virus; UV for ultraviolet; $\bar{V}$ for partial specific volume; μ for electrophoretic mobility; η for viscosity.

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(see also 28, 86, 227, 230, 328, and "New Preparations")

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CARBOHYDRATE METABOLISM[1,2]

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The past year has seen important advances in various aspects of carbohydrate metabolism. Because of limitations of space, this review is confined to developments in those areas where interpretation in terms of chemical mechanisms can be made, including polysaccharide formation and degradation, glycolysis, alternate pathways of carbohydrate metabolism, carbon metabolism in photosynthesis, tricarboxylic acid cycle reactions and some physiological effects related to specific biochemical reactions. Because of these arbitrary limitations, many meritorious papers could not be included and, in addition, the important studies on α-keto acid and succinate oxidation have been omitted, since these subjects are covered in another chapter of this volume.

POLYSACCHARIDES

Synthesis of Disaccharides

Sucrose.—The synthesis of sucrose has been demonstrated to occur by at least two distinct pathways. In addition to sucrose phosphorlyase of bacteria, an enzyme from wheat germ was shown by Leloir & Cardini (1) to catalyze a comparable transfer of glucose from UDPG[3] to fructose, forming reversibly sucrose and UDP.[3] The system is specific for free fructose, and does not react with either fructose phosphates or unphosphorylated sorbose, aldoses, or arabinose. Sucrose phosphorylase has been purified by Weimberg & Doudoroff (2) from three microorganisms: *Pseudomonas saccharophila*, *Pseudomonas putrefaciens*, and *Leuconostoc mesenteroides*. The purified preparations exhibit very similar properties, but differ in reactions with certain sugars: the enzyme from *P. putrefaciens* does not donate glucosyl groups to L-sorbose or D-xylulose, and the Leuconostoc enzyme is less inhibited by glucose than the others. The preparations do not catalyze the exchange of glucose of UDPG, and no chromatographic evidence for any other stable glucose intermediate could be found. The hydrolytic activity previously observed in sucrose phosphorylase preparations occurs in constant ratio to the

[1] This review covers approximately the period from October, 1953 through October, 1954.

[2] The following abbreviations are used: AMP for adenosinemonophosphate; ADP for adenosinediphosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); IDP for inosinediphosphate; ITP for inosinetriphosphate; ThPP for thiamine pyrophosphate; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form); UMP for uridinemonophosphate; UDP for uridinediphosphate; UTP for uridinetriphosphate; UDPG for uridinediphosphate glucose.

phosphorolytic activity and is therefore considered to be an intrinsic property of the enzyme.

Isotopic experiments with Canna leaf preparations reported by Putman & Hassid (3) indicate that sucrose is formed *in vivo* by a mechanism involving phosphorylated hexoses, as previously found by Calvin & Benson (4) with other plants. Both glucose and fructose labels are distributed equally in the two halves of sucrose. The evidence against the participation of free fructose argues in favor of a third mechanism of sucrose formation, perhaps involving sucrose phosphate, which was isolated from plant preparations by Buchanan (5). Detached tobacco leaves were found by Vittorio, Krotkov & Reed (6) to incorporate isotope from glucose and from glucose-1-phosphate into both halves of sucrose, while little label appeared in free fructose. A sucrose-forming system was also detected in potato macerates by Schwimmer & Makower (7). They found no net increase when glucose, ATP^3, and inorganic phosphate were added to the preparations, but the addition of dextran sucrase of *L. mesenteroides* resulted in the accumulation of dextran, presumably formed from sucrose. Preliminary reports by Turner (8, 9) described the formation of sucrose from glucose-1-phosphate and fructose in pea extracts by an unknown mechanism.

Trehalose disaccharides.—Leloir & Cabib (10) found an enzyme in brewers' yeast which transfers a glucosyl group from UDPG to glucose-6-phosphate, forming trehalose phosphate.

Enzymes that Attack Oligosaccharides

Transfructosidases.—During the last year there have been many examples of transfer of fructose residues by invertase and similar enzymes from various sources. These enzymes, formerly recognized only as hydrolytic, appear to be glycosyl donors which utilize a variety of acceptors.

Edelman (11) has studied exchange and transfer reactions catalyzed by sucrase preparations from yeast and from several molds. These enzymes, formerly thought to be either fructosidases or glucosidases because of differences in apparent inhibition by fructose and glucose, are now all shown to transfer fructofuranose groups, although they differ in their specificities for various acceptors. The mold enzymes are capable of exchanging the glucose residue of sucrose, whereas the yeast enzyme transfers fructose only to positions of glucose other than C-1. Two invertase preparations were shown by Kurasawa *et al.* (12) to vary greatly in relative reaction with fructose acceptors. An enzyme from *Aspergillus oryzae* transfers fructose to various sugars but not to methanol, whereas an enzyme from rice transfers readily to methanol, but not to sugars.

Bacon (13) isolated two disaccharides other than sucrose and three trisaccharides from incubation mixtures of yeast invertase and sucrose. Gross and collaborators (14, 15) have identified two such trisaccharides as kestose, in which fructose is transferred to the 6-position of the fructose component of sucrose, and neo-kestose, in which the 6-position of the glucose component

accepts the fructose. Many compounds detected by paper chromatography remain to be identified; electrophoretic separation has revealed additional products.

Pazur (16) has continued studies with mold enzymes in which naturally-occurring fructose polysaccharides were tested as substrates and acceptors (cosubstrates). Inulobiose, in which the glycosyl bond involves position 1 of fructose, serves as both donor and acceptor, yielding probably free fructose and inulotriose. Stachyose, a tetrasaccharide in which the glucose portion of sucrose is substituted in position 4 with a galactose disaccharide, does not serve as a donor, but does accept fructose to form a compound which migrates on paper like a pentasaccharide. Plantose, an isomer of raffinose in which the fructose portion of sucrose is also condensed with galactose, is neither a substrate nor acceptor.

Transfructosidation had been proposed by Dedonder (17) and by Bacon & Edelman (18) as a mechanism of fructose polysaccharide synthesis in plants. Support for this hypothesis was presented by Ash & Reynolds (19), who isolated ketose oligosaccharides with single glucose residues from apricot fruit and by Harwood, Laidlaw & Telfer (20), who isolated from rye grass short-chain fructosans, terminated with 1-linked glucose, determined by methylation. These findings indicate that sucrose serves as a primary fructosyl acceptor leading to the formation of an inulin-type polysaccharide.

The levansucrase of *Bacillus subtilis* appears to be inhibited by glucose in the medium (21). Since the level of this adaptive enzyme is not influenced by glucose (22), it is probable that glucose can serve as acceptor for this fructose transferring enzyme. A transfructosidase with a pH optimum of 5.6 to 5.8 was partially purified from stem juice of *Agave vera cruz* (23).

The invertase of yeast cells was localized at the cell surface by studies reported by Demis, Rothstein & Meier (24). They found that inhibition by uranyl ions is reversed by an extracellular concentration of inorganic phosphate only 1 per cent of the intracellular, and that the hydrolytic products accumulate in the medium. Mandels (25) has presented indirect evidence for a mechanism of sucrose utilization by spores of *Myrothecium verrucaria*, which does not involve invertase. The spores do contain a typical fructosidase, but this also appears to be at the surface and is inactivated by acid. Acid treatment inactivates several other polysaccharide-hydrolyzing enzymes of the spores (26). Acid-treated spores do assimilate and oxidize sucrose, although invertase cannot be demonstrated at comparable levels. Intact spores also metabolize sucrose more rapidly than the constituent monosaccharides, and sucrose metabolism is inhibited somewhat more than that of other sugars by arsenate. Therefore, although a direct demonstration could not be made, Mandels suggested that a sucrose phosphorylase is involved.

The level of the adaptive invertase of *Saccharomyces fragilis* was shown by Davies (27) to be determined only by the sugar concentration in the medium. Since yeast growing under conditions of short mean generation time used sucrose more rapidly than could be accounted for by invertase

assays, he concluded that another mechanism for sucrose utilization exists. However, this conclusion is based on relatively small discrepancies, and direct evidence for the existence of an alternate pathway is needed.

Invertase appears to be lacking in the intestine of newborn pigs. The absence of this enzyme was inferred from feeding experiments by Becker *et al.* (28), which confirmed the earlier report of Johnson (29) that sucrose does not support growth in these animals, although invert sugar can be utilized for growth.

Transglucosidases.—Tsuchiya & Stringer (30) have investigated the substrate specificity of dextransucrase from *L. mesenteroides*. They found that the same products are formed from dextran or isomaltose as donor as from sucrose. Fructose as an acceptor forms a disaccharide leucose, which is also a glucosyl donor. Presumptive evidence for a transglucosidase in the nectar of *Robinia pseud-acacia* was reported by Zimmerman (31), who found that free fructose was formed from sucrose without a corresponding formation of glucose.

Maltose and other α-glucosides participate in transfer reactions. Giri *et al.* (32) have shown the conversion of maltose to isomaltose by an enzyme in the culture filtrate from *Penicillium chrysogenum*. Pazur (33) has shown that a transglucosidase from *A. oryzae* can form isomaltose from glucose and dextrotriose; this demonstrates a reversal of the earlier synthesis of dextrotriose from isomaltose by this enzyme. Ishizawa & Miwa (34) separated yeast invertase, which transfers fructose from maltase, which transfers glucose from sucrose.

Transfer of glucose residues was involved by Blair & Pigman (35) in an explanation of a complicated problem in yeast fermentation. Maltose is not fermented completely unless high concentrations of glucose are present, but glucose added later does not complete the utilization of maltose. The explanation offered is that in the absence of glucose a polymer accumulates with increasing chain length. Glucose serves as glucosyl acceptor and permits reversal of the polymerization in the early stages. When the polymer is very large, reversal is slow. This mechanism for the utilization of maltose appears to offer an explanation which can reconcile the positions of those who support a unitary scheme for fermentation and those who advocate a separate mechanism for oligosaccharides, since transfer reactions may result in the rapid utilization of oligosaccharides with concomitant liberation of free hexoses.

The Schardinger dextrins also serve as glycosyl donors. An amylase from *Bacillus macerans* uses any of a number of sugars as acceptor for the start of chains of varying numbers of glucose residues (36). All of these reactions appear to be reversible, as eventually an equilibrium mixture of chains is established.

A novel group of glucosyl acceptors was found by Whitby (37). He showed transfer of glucose from maltose, maltulose, or turanose, but not from cellobiose, lactose, or α-methyl glucoside, to riboflavin and certain other isoalloxazine derivatives. The glucosides are more water-soluble than the original flavin.

Transgalactosidases.—The transfer of galactose residues was studied by Wallenfels, Bernt & Limberg (38) with enzymes from mold, snails, bacteria, and serum. All of the enzymes are β-galactosidases which transfer from lactose to a variety of acceptors, including glucose, galactose, and lactose, as well as to water. The enzymes are all very similar, but differ in relative substrate affinities. Similar results were obtained by Pazur (39) with a yeast enzyme. Wallenfels *et al.* indicated that lactose was not formed from lactobiose, and C^{14} incorporation studies by Pazur show very little isotope in the lactose area of paper chromatograms. It is apparent from these results that lactose cannot by synthesized *in vivo* by a nonspecific β-galactosidase. On the other hand, it must be assumed that the action of the enzyme is not irreversible, but that other positions than the 4-position of glucose are favored acceptor sites for either steric or thermodynamic reasons. The cases reported above seem to indicate that primary alcohol groups are preferentially used as acceptors by transglycosidases.

Stachyose contains two α-galactosidic linkages. Courtois *et al.* (40) have shown that coffee α-galactosidase removes one residue at a time, forming raffinose as an intermediate which is split to galactose and sucrose.

An enzyme from mold was shown by Weinland & Nüchterlein (41) to behave like a snail enzyme in splitting both D- and L-galactose from galactogen.

A novel concept of lactose hydrolysis was suggested by Fournier (42). He attributes a "remarkable effect of lactose on ossification" to production of β-galactose, which is used before it mutarotates since fed galactose is largely excreted. A number of alternative explanations might be considered, however, including differential rates of absorption, differential distribution in the animal, and transfer reactions of galactosidases. Against the mutarotation argument is the fact that at equilibrium the concentration of β-galactose exceeds that of the α-isomer.

Synthesis and Degradation of Polysaccharides

Phosphorylase.—Potato phosphorylase was shown by French & Wild (43) to be capable of using a three-residue chain of glucose units as a primer, although chains of four or more residues are much more efficiently used. It was also found that no reducing group is needed in the primer. By inhibiting α-amylase and phosphatase with $HgCl_2$ and NaF, Porter (44) was able to obtain equilibrium measurements with potato phosphorylase. As previously established for muscle phosphorylase, the equilibrium is independent of primer, but depends only on glucose-1-phosphate and inorganic phosphate. The average chain length therefore is a function of the amount of primer added.

A study of the concentrations of sugar phosphates and inorganic phosphate in yeast was made by Trevelyan, Mann & Harrison (45). They found concentrations of glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate in fermenting yeast consistent with the phosphoglucomutase and phosphohexose isomerase equilibria. The ratio of inorganic phosphate to

glucose-1-phosphate, however, was much higher than expected from the phosphorylase equilibrium. The suggestion was made that a phosphate gradient exists inside the cell and that equilibrium is maintained in the environment of the enzyme. An alternative explanation might consider the possibility that a large part of the "inorganic" phosphate within the cell is so bound to proteins, metals, etc. that the effective concentration is much less than indicated by analysis.

It has been shown by Larner (46) that phosphorylase acts at random in a multichain mechanism, rather than by degrading single chains completely. This was shown in a direct fashion made possible by a specific analysis for completely degraded chains. Chains which have been degraded to the phosphorylase limit yield glucose when subjected to amylo-1,6-glucosidase. Whereas the single chain mechanism requires that free glucose be liberated by the glucosidase in proportion to phosphorylase action, partially degraded polysaccharides subjected to the glucosidase yielded essentially no free glucose.

The structure of glycogen has been well established in previous years. In view of the well-accepted structure, it is difficult to interpret the claim of Laszt (47) that not only do physical differences exist between normal and diabetic liver glycogen, but that the diabetic glycogen is more labile to acid. Bell & Manners (48) have reported further confirmation that glycogen contains only 1,4 and 1,6 linkages. Glycogen and dextrin samples treated with periodate yielded no glucose on hydrolysis. Any glucose residues substituted at positions 2 or 3 would have resisted periodate oxidation.

A kinetic analysis of liver and muscle glycogen has been made by Stetten & Stetten (49, 50). With C^{14}-labeled glucose, they measured the incorporation of isotope in various tiers of the isolated glycogen by alternate degradation with β-amylase or phosphorylase and amylo-1,6-glucosidase. Their results showed that glycogen is inhomogeneous, with the outer tiers exchanging glucose residues more rapidly than inner. In liver the turnover of the inner tiers is much more rapid than in muscle.

The widespread occurrence of glycogen, and therefore of the enzymes which synthesize it, in microorganisms was demonstrated by Levine *et al.* (51), who isolated glycogen from several strains of enteric bacteria. They found the glycogen content to reach 48 per cent of the dry weight of the cells grown on appropriate media.

The pattern of development of enzymes and glycogen in the fetus of guinea pigs was studied by Nemeth and collaborators (52). It was found that phosphorylase, phosphoglucomutase, hexokinase, and phosphorylase primer were all present in the liver for several days before glycogen could be demonstrated. It was suggested that the deficiency is in branching enzyme, which was not assayed. In the absence of more evidence about the activity of systems competing for glucose phosphate, alternate explanations for the failure of younger livers to form glycogen must be considered. Nemeth (53) also made the interesting observation that glucose-6-phosphatase is lacking

in fetal livers and relates this to the accumulation of glycogen late in fetal development. However, the accumulation of glycogen depends upon many reactions, any of which might be limiting, and an explanation for the physiological phenomenon must evaluate these factors as well.

A surprising contention has been advanced by Boser (54), who claims to have evidence that the hormone glucagon is identical with the enzyme phosphorylase. It will be of interest to see the data on which this claim is based.

Amylases.—In an extensive series of publications, Myrbäck and associates (55 to 59) reported on experiments with both α- and β-amylases. It was found that the carboxylic acid produced by hypoiodite oxidation of a hexasaccharide was hydrolyzed as rapidly as the original hexasaccharide by malt β-amylase, half as fast by salivary amylase, and very slowly by malt α-amylase. Salivary and β-amylase attack the hexasaccharide as rapidly as starch, whereas the malt α-amylase rate with starch is 6 times faster. The formation of free glucose is attributed to hydrolysis at the reducing end of the chain by α-amylase, which produces no free glucose from the acid analogue. The other enzymes do not produce free glucose under the conditions studied.

α-Amylases.—It is interesting to note that, unlike the enzymes which split smaller oligosaccharides, the amylases have not been shown to catalyze transfer reactions. The analysis of the products of hydrolysis therefore seems to be a valid means for determining the nature of the enzyme action. Myrbäck reported that salivary amylase yields predominantly maltose from starch, but in addition free glucose and dextrin are also formed. The major dextrin was tentatively identified as a tetrasaccharide with one α-1,6 linkage. The formation of the free glucose was attributed in part to a small amount of maltase and in part to the amylase, although the possible existence of other enzymes was not eliminated from consideration. It appears that the tetrasaccharides which Myrbäck found resistant to amylases were all natural products containing isomaltose linkages. This is consistent with the findings of Pazur (60) that salivary amylase will attack not only maltotetraose, but even maltotriose. In the case of the triose, the products are glucose and maltose. The tetraose is preferentially split in the middle to form maltose, but a certain amount of triose and glucose are formed by an asymmetric split. The crude enzyme used did not hydrolyze maltose.

Differences in the mechanism of action of amylases from pancreas, *B. subtilis*, and barley were indicated in studies by Blom & Schwartz (61). They found that the changes in optical rotation caused by the various enzymes did not correspond to the increase in reducing power.

The mechanism of action of α-amylases has been studied by Bird & Hopkins (62). They determined the products of hydrolysis of various substrates by α-amylases of malt, *B. subtilis*, saliva, and *A. oryzae*. The ability of the various enzymes to hydrolyze bonds near the reducing end of a chain was determined by comparing the products obtained from a partially degraded amylopectin with those from amylose degraded to have a similar number of

nonreducing end groups. From the appearance of maltose or maltotriose among the early fission products of amylose degradation but not from amylopectin, it was deduced that salivary and malt amylases attack the second linkage from the reducing end, and bacterial amylase the third. The formation of tri- and tetrasaccharides from both types of substrate by salivary amylase indicates an inability to attack the two linkages adjacent to the nonreducing end of a chain. Similarly, the formation of negligible traces of chains smaller than six units by bacterial or malt enzymes indicates attack by these enzymes only beyond the fifth linkage from the nonreducing end. Between the limits described above, it is suggested that attack is random. From these and similar observations, a picture of enzyme substrate interaction is drawn which proposes that each enzyme has sites for binding the number of units relatively immune to attack at each end of the chain. Chains long enough to fit over the entire binding zone are split rapidly. Smaller chains are held more weakly and consequently split with more difficulty, releasing smaller products. This theory explains the rapid formation of oligosaccharides of sizes characteristic of each enzyme, followed by slower hydrolysis of compounds as small as maltotriose. Experiments reported by Seiji (63) show that α-amylase attacks β-amylase limit dextrin in the same manner that it attacks amylopectin. He concluded, therefore, that α-amylase is not influenced by 1,6-linkages at the nonreducing ends of chains.

It is apparent that α-amylase hydrolyzes polysaccharides beyond the branch points which limit β-amylase and phosphorylase. The time sequence of hydrolysis of inner and outer chains was studied by Lohmar (64), who isolated the polysaccharides remaining after brief α-amylase digestion. The relative amounts of reducing power, formate produced by periodate, and groups available to β-amylase led to the conclusion that both inner and outer chains are reduced in size and, therefore, that both inner and outer branches are attacked.

Campbell (65) has succeeded in crystallizing α-amylase from a thermophilic bacterium grown either at 35°C. or at 55°C. Each preparation appears to be homogeneous by electrophoretic and solubility criteria. The two preparations differ, however, in that the crystals from the cells grown at the higher temperature are more heat stable. This very interesting study is an encouraging approach to determining the changes an organism can make in an enzyme in response to an altered environment and the factors responsible for heat stability.

Thayer (66) has studied the adaptive amylases of *P. saccharophila*. At least two amylases are produced in response to a number of polysaccharides, one intracellular and one extracellular. An α-amylase was purified from the medium. It is not clear how many different amylases occur within the cells. At least one is noteworthy in that it is induced by cellobiose.

β-Amylase.—Both soy bean and crystalline sweet potato β-amylase were reported by Hopkins & Jelinek (67) to attack chains of polysaccharides in random fashion. They reported similar affinities for long and short chains,

with the result that random attack causes a more rapid disappearance of the short chains from a mixture. They argued strongly for a "multichain" action by β-amylases, as opposed to a mechanism which involves complete hydrolysis of one chain at a time. They also reported that the crystalline sweet potato enzyme accumulates a tetrasaccharide product but is capable of hydrolyzing maltotriose slowly.

It has been known that β-amylase causes a decrease in rotation. However, Bird & Hopkins (68) found that immediately after mixing enzyme and any of a variety of substrates the rotation was higher than expected. It has been established that β-maltose is the initial product and that this gradually mutarotates to the α, β-mixture with a higher rotation. In the cases studied, however, treatment with alkali to stop enzyme action and increase mutarotation led to a decrease in rotation. The increased rotations of the enzyme substrate mixtures were interpreted as indications of enzyme-substrate compounds. Alternate explanations are not obvious, but it is very difficult to visualize an enzyme-substrate interaction of such proportions that a few milligrams of enzyme increases perceptibly the rotation of gram quantities of polysaccharides.

The β-amylase of wheat has been crystallized by Meyer, Spahn & Fischer (69). The crystalline enzyme represents a 15-fold purification from an aqueous extract of flour. The purification includes acidification to pH 3.7 which destroys the α-amylase.

Amyloglucosidase.—Phillips & Caldwell (70) had previously reported the purification from *Rhizopas delemar* of an enzyme which produces free glucose from polysaccharides without the appearance of any intermediate oligosaccharides. The crystallization of this enzyme, "gluc amylase," has been reported by Fukumoto, Sakazaka & Minamii (71). Weill, Burch & Van Dyk (72) purified a similar enzyme, amyloglucosidase, from *Aspergillus niger*. The only reducing sugar formed by this enzyme is β-glucose. The purification of an amyloglucosidase from *A. oryzae* was described by Drews, Specht & Olbrich (73).

1,6-Glucosidases.—The hydrolysis of dextran by an adaptive enzyme from various *Penicillium* species was found by Kobayashi (73a) to liberate β-isomaltose and β-isomaltotriose by random attack. The enzymes are sensitive to I_2 and heavy metals and have pH optima between 5.25 and 6.5. An enzyme from intestinal mucosa was found by Seiji (74) to attack α-amylase limit dextrin. This enzyme was independently purified by Larner & McNickle (75), who named the enzyme oligo-1,6-glucosidase. It hydrolyzes oligosaccharides which are not hydrolyzed by amylo-1,6-glucosidase of muscle. This intestinal enzyme splits compounds as small as isomaltose and panose. On the other hand, it does not attack phosphorylase limit dextrins.

Levan polyase.—The synthesis of levan from sucrose is an essentially irreversible process. The hydrolysis of levans to oligofructosides has been shown by Hestrin & Goldblum (76) to be catalyzed by an enzyme, levan polyase, found in *Azotobacter chroococcum* and *Bacillus asterosporus*. The

products of polyase hydrolysis are attacked by invertase and similar fructosidases which do not attack levans.

Cellulose.—Two groups have reported on incorporation of labeled sugars into cellulose. Both wheat plants (77) and *Acetobacter xylinum* (78) appear to incorporate glucose directly. Sorbitol appears to be split to trioses before it is built into cellulose by wheat. Also, the label of 1-C^{14}-mannitol is distributed in both positions 1 and 6 of the glucose in cellulose synthesized by *Acetobacter* (79). The utilization of glucose for cellulose formation by *Actobacter* is increased by addition of ethanol to the medium. However, neither ethanol nor acetate carbon atoms are incorporated into cellulose (80).

Hestrin & Schramm (81) have studied cellulose synthesis by nonviable cells of *Acetobacter xylinum*. They used washed, lyophilized cells which contain only traces of cellulose and retain the ability to form cellulose from glucose in the presence of air. The product was identified as α-cellulose. Barclay *et al.* (82) claimed to have obtained synthesis of cellulose with insoluble fragments of *Acetobacter acetigenum*. The polysaccharide produced by this organism was identified as cellulose by chemical and physical methods.

The degradation of cellulose by extracts of *A. niger* proceeds completely to glucose, but cellobiose appears to be an intermediate (83). Enzymes which yield glucose from cellulose are produced by *Neurospora crassa* grown at 35°C. (84). The cellulase of *Myrothecium verrucaria* is extremely heat-resistant (85). It hydrolyzes cellulose, rayon, cellodextrins, and oligosaccharides as small as cellotriose. The principal products are cellobiose and glucose, since cellobiose is split very slowly. A heat-labile cellobiase is also present in the culture medium, which appears to catalyze transfer reactions as well as hydrolysis. Whole cells of *A. niger* (86) and a dialyzed extract of *Aspergillus flavus* (87) were found to carry out transfer reactions with cellobiose. Cell-free preparations from *Chaetomium globosum* were found to form cellotriose and higher polymers from cellobiose (88). All of the cellobiose was consumed in oligosaccharide formation when notatin was added to remove the free glucose formed. By means of a column technique for fractionally dissolving ammonium sulfate precipitates, van Sumere (89) has obtained fractions from barley enriched with respect to cellulase and other fractions containing hemicellulase free from cellulase.

Polysaccharides containing N-acetyl glucosamine.—Schiller, Mathews & Dorfman (90) have measured the rate of biosynthesis of mucopolysaccharides in rabbit skin. They found incorporation of radioactive acetate into both hyaluronic acid and chondroitin sulphuric acid. The decay curve indicated a half-life of about two days for hyaluronic acid and seven to eight days for chondroitin sulphuric acid. Since the latter value is similar to that obtained previously by Böstrom & Gardell (91) with S^{35} in rats, it seems likely that these values represent turnover of the entire molecule, not of single components. Roseman *et al.* (92) studied the incorporation of 6-C^{14}-glucose into the hyaluronic acid of Group A *Streptococci*. The failure of the label to randomize to other positions in either the glucuronic acid or glucosamine indicates a direct conversion of glucose to these compounds.

The hydrolysis of umbilical cord hyaluronic acid by testicular hyaluronidase was shown by Weissmann *et al.* (93) to yield a mixture of oligosaccharides. Seven compounds were isolated and identified as members of a series formed from disaccharide units. The repeating unit is glucuronidoacetylglucosamine, identical with the N-acetyl derivative of hyalobiuronic acid, isolated from acid hydrolysates. The major product of exhaustive hydrolysis of hyaluronic acid by testicular hyaluronidase is a tetrasaccharide. Enzymes from Pneumococcus, Streptococcus, Staphylococcus, or Clostridium welchii form a disaccharide from hyaluronic acid (94). The disaccharide contains glucosamine, but the uronic acid portion is modified, apparently by the formation of a double bond to form an α, β-unsaturated acid. The unsaturation appears to be involved in the splitting of the polysaccharide, since the bacterial enzyme splits the tetrasaccharide produced by the animal enzyme to equal amounts of N-acetylhyalobiuronic acid and the "bacterial disaccharide." This observation makes it tempting to speculate that the bacterial enzyme does not hydrolyze but splits by elimination of the aldehyde from the acetal linkage, leaving a double bond in the aglycone residue.

An enzyme from lobster and a similar one from *A. oryzae* were found by Kuhn & Tiedemann (95) to hydrolyze β-glucosides of N-acetyl glucosamine. The enzymes have broad pH optima near 4 and attack a variety of synthetic β-glucosides, including the methyl, ethyl, propyl, and benzyl derivatives of N-acetylglucosamine. The enzymes attack N,N′-diacetyl chitobiose and one of the lactobioses obtained from milk. Other milk polysaccharides, a lactobiose and lactotetraose, were not attacked. At high concentrations of the products [N-acetyl glucosamine (5 per cent) and ethanol (15 per cent)], reversal could be detected by the appearance of β-ethyl-N-acetylglucosaminide on paper chromatograms.

Enzymes from *Proteus vulgaris* studied by Konetzka, Pelczar & Burnett (96) split sulfate and ammonia from chondroitin sulfuric acid in addition to releasing reducing power. Inhibitors differentially influence sulfate and ammonia release, which appear to be secondary to the appearance of reducing groups.

Bostrom *et al.* (97, 98) studied the exchange of S^{35} sulfate into chondroitin sulfuric acid by cartilage slices. They found the exchange to be stimulated by glucose, and more by a heat-stable, dialyzable factor from liver, and to be inhibited by cortisone and sulfhydryl-binding reagents.

An adaptive chitinase from *Streptomyces* was found by Reynolds (99) to form N-acetylglucosamine and N,N′-diacetyl chitobiose. A preparation from snail intestine was found by Hackman (100) to hydrolyze chitin and its sulfate ester to glucosamine and N-acetylglucosamine.

The existence in several bacterial species of polysaccharides which serve as substrates for testicular hyaluronidase was shown by Warren *et al.* (101, 102). It was concluded from studies on lysozyme-treated *Sarcina* that the polysaccharide is intracellular.

Hyaluronidase was shown to be one of an increasing group of enzymes

known to be reversibly inactivated by heat. In simultaneous studies in two laboratories (103, 104) it was shown that the inactive enzyme produced by treatment with dilute acid at 100°C. for as long as an hour recovers a major part of its activity over several hours or days at 0°C. The reactivation is a function of pH as well as of temperature. The parallel destruction and recovery of activity measured with hyaluronic acid and chondroitin sulfuric acid is evidence that both substrates are hydrolyzed by a single enzyme.

The hydrolysis of N-acetyl glucosamine was found by Roseman (105) to be catalyzed by an enzyme from *E. coli*. The enzyme attacks N-acetyl-D-galactosamine at 1/10 the rate for N-acetylglucosamine and does not hydrolyze acetyl amino acids.

Bifidus factor.—The nature of the growth factor for *Lactobacillus bifidus* has been elucidated to a great extent. This factor was found in human milk (106), in stools from breast-fed babies (107), and in hog gastric mucin (108). The activity is not restricted to one compound but depends on the existence of particular groupings within a polysaccharide. Following the determination that all active materials contained N-acetylglucosamine (106), Rose *et al.* (109) found that β-methyl-N-acetyl-D-glucosaminide, but not the α-isomer, stimulated the growth of *L. bifidus*. The α-glycoside potentiated the effect of the β-, possibly by decreasing the rate of enzymatic hydrolysis. A crystalline *bifidus* factor was isolated from hog gastric mucin by Tomarelli *et al.* (108) and tentatively identified as galactosyl acetyl glucosamine. In addition to the compounds mentioned above and mildly hydrolyzed chitin, Zilliken *et al.* (110) found weak activity in acetylglucosamine. An enzyme from *L. bifidus* was found to transfer galactose from lactose to N-acetylglucosamine to form two compounds, one of which was identical with the compound isolated by Tomarelli *et al.* The other isomeric compound has relatively little activity as a growth factor (110). Certain sources of *bifidus* factor were reported to promote the growth of weanling rats, but other preparations active for bacteria had no effect on rats (111).

Polygalacturonides.—The nature of a yeast polygalacturonase has been studied extensively by Phaff and his collaborators. They found that the enzyme accumulates in the medium and is not accompanied by pectinesterase (112). The enzyme was purified 20-fold to a point where the major electrophoretic component showed activity but other proteins were still present (113). An interesting step in the purification involved adsorption on an insoluble pectic acid gel at pH 3 and elution at pH 5 (114). This enzyme has been shown to hydrolyze pectic acid rapidly to small oligosaccharides. The rate of hydrolysis is much slower with tri- and tetragalacturonic acids (114, 115). Digalacturonic acid is not hydrolyzed and is not bound to the enzyme, since it does not inhibit hydrolysis of larger molecules as trigalacturonic acid does. Yeast polygalacturonase also hydrolyzes pectin to a slight extent (116), consistent with the hypothesis of Jansen & MacDonnell (117) that adjacent carboxyls are necessary to provide a site for hydrolysis. No evidence was found for more than one enzyme attacking polygalacturonic acids.

A purified citrus polygalacturonase was studied by McCready & Seegmiller (118), who prepared a number of synthetic methyl esters of oligogalacturonic acids. Their evidence shows that this enzyme also requires adjacent carboxyl groups. Unlike the yeast enzyme, the citrus enzyme attacks digalacturonic acid, although slowly. The position of the carboxyl group is not absolutely critical, since it was found that galacturonosyl galactonic acid is also hydrolyzed.

McCready & McComb (119) studied the kinetics of polygalacturonic acid hydrolysis by mold enzymes. The ultimate product, galacturonic acid, and di- and trigalacturonic acids were crystallized as brucine salts. Using ion exchange chromatography Derungs & Deuel (120) have isolated mono-, di-, tri-, and tetragalacturonic acids obtained by hydrolysis of pectic acid by a mold enzyme. The digalacturonic acid was shown by methylation studies of Altermatt & Deuel (121) to be 4-(α-D-galacturono-pyranosido)-D-galacturonopyranose. Essentially the same results were obtained by Jones & Reid (122) who isolated oligosaccharides produced from apple pectic acid by preparations from *Aspergillus foetidus* and anaylzed the methylated disaccharide.

Other polysaccharides.—The polyribophosphate from the capsule of *Hemophilus influenzae* has been obtained in a highly polymerized form by Zamenhof & Leidy (123). The viscosity of solutions of the highly purified compound was reduced by ribonuclease, which previously had been known to attack only derivatives of pyrimidine nucleotides.

The isolated cell walls of several bacteria were found by Salton (124) to be dissolved by crystalline egg white lysozyme. The products were polydisperse with major components having molecular weights in the order of 10,000 to 20,000. No glucose was liberated. A dialyzable compound resembling N-acetylglucosamine was found.

O^{18} experiments.—The mechanism of group transfer reactions was reviewed by Koshland (125). In agreement with his generalization about the cleavage point in transfer reactions, H_2O^{18} experiments showed that sucrose is split between fructose and the bridge oxygen (126). Bunton *et al.* (127) similarly found O^{18} to be incorporated into the sugar produced when α- and β-methyl glucosides were hydrolyzed either by acid or by appropriate enzymes, yeast α-glucosidase, or almond emulsin.

GLYCOLYSIS

General Considerations

Occurrence of glycolytic systems. It has been established that glycolysis is carried out by a number of tissues from various organisms. Rathbone & Rees (128) found that extracts of tissues from *Ascaris lumbricoides* convert pyruvate to lactate and that no pyruvate is oxidized. Several of the glycolytic enzymes were demonstrated. Similarly, all of a number of glycolytic enzymes searched for were found in the crystalline lens of the calf by Mandel & Israelewicz (129) and in the ciliary processes by DeRoeth (130). Glycolytic

enzymes, glucose-6-phosphate dehydrogenase, and 6-phosphogluconic dehydrogenase were found in *Achromobacter fischeri* by Friedman (131). Isotopic studies by Pine, Haas & Barker (132) are consistent with a glycolytic pathway in *Butyribacterium rettgeri* yielding DL-lactate.

This pathway also accounts for glucose metabolism in *Clostridium perfringens* (133) although anomalous differences in labeling of ethanol and acetate were observed. Evidence for a glycolytic mechanism has also been obtained with *Asterococcus mycoides* (134), which yields extracts containing hexokinase and aldolase.

Influence on glycolysis. It was reported by Hockenhull *et al.* (135) that *Streptomyces griseus* oxidizes glucose to CO_2 and also oxidizes compounds of the tricarboxylic acid cycle. The existence of a conventional glycolytic route for the oxidation of glucose was indicated by a Pasteur effect, in which pyruvate and lactate accumulated when glucose was metabolized in the presence of limited amounts of oxygen. An inactivation of yeast fermentation by complete anaerbiosis was shown by Wikén & Richard (136) to be reversed by traces of oxygen. Studies by Miller & Olson (137) showed a differential effect of fluoride on the dissimilation of pyruvate and lactate by cardiac muscle. The metabolism of lactate is therefore considered to proceed by a pathway excluding pyruvate, and a relation to propanediol metabolism is indicated. Volk (138) found that cells of *Propionobacterium pentosaceum* grown in fluoride become impermeable to this ion and also lose the ability to attack phosphorylated substrates. However, nonphosphorylated carbohydrates are fermented, probably by a normal glycolytic mechanism, since extracts of these cells contain a normal amount of fluoride-sensitive enolase. These results remove the basis for consideration of an alternate scheme for carbohydrate metabolism in the presence of fluoride. An anomolous result was obtained by Brady & Duggan (139), who found that high concentrations of azide permitted fermentation of endogenous carbohydrate of baker's yeast, which under ordinary circumstances cannot be fermented.

Clowes & Keltch (140) found glucose, mannose, and fructose to be glycolyzed by cells of Ehrlich's ascites carcinoma. Glucose, but not mannose or fructose, fermentation was found to be stimulated by dinitrocresol. Galactose, xylose, and arabinose were not fermented.

McIlwain (141) has found that glucose oxidation by cerebral tissues is increased by electrical stimulation. Parallel studies by Heald (142) with inhibitors showed that iodoacetate and fluoride at given concentrations had greater effects on stimulated than on unstimulated oxygen consumption. The results of studies with these inhibitor and malonate and glucose or lactate as substrates indicate that triosephosphate dehydrogenase and enolase can be made limiting, and that pyruvate oxidation in this tissue competes successfully with reduction to lactate when substrate is limiting.

A genetic agent in *Pneumococcus* which produces cells unable to oxidize lactate was described by Ephrussi-Taylor (143). These cells oxidize glucose slowly, and in unaerated cultures produce high yields of lactic acid. An in-

teresting interaction of metabolic products was found in Neurospora mutants by Strauss & Pierog (144). A slow-growing mutant appears to be inhibited in growth by the accumulation of acetaldehyde and ethanol. An additional mutation (suppressor) which results in decreased carboxylase activity permits better growth by limiting the accumulation of the toxic products. This study also included interesting accounts of large variation of the levels of aldolase, glucose-6-phosphate dehydrogenase, and alcohol dehydrogenase with varying growth conditions.

The inhibition of yeast growth and glucose oxidation by hexachlorocyclohexanes was found by Barnes & Sagar (145) to be a complex phenomenon, not easily explained by analogue inhibition. Growth was more sensitive to the δ-than to the γ-isomer, and the sensitivity of various strains could be related to their inositol requirements. On the other hand, glucose fermentation was inhibited only by the δ-isomer, and the inhibition was not antagonized by inositol. In one yeast oxygen consumption was decreased more by the δ-than by the γ-isomer and only the effect of the γ-isomer was reversed by inositol.

Requirements of rat and mouse tissue homogenates for cofactors to support carbohydrate oxidation were studied by Wenner, Dunn & Weinhouse (146). Various tissues differ in the extent of their requirement for added DPN[2] to achieve maximum rates of oxidation. The effect of adenine nucleotides varies from stimulation to inhibition with DPN addition. With appropriate ions, tonicity, and cofactors, either glucose or fructose can be oxidized twice as fast by homogenates as by slices. Hickman & Ashwell (147) found that the decreased anaerobic glycolysis found in homogenates of spleen from x-irradiated mice was limited by adenylic acid, which in this tissue was converted to adenosine, inosine, and hypoxanthine.

A metabolic interrelationship with practical implications was studied by Vitale *et al.* (148). This group found that oxidation of ethanol by rats and its removal from circulation was inhibited by administration of pyruvate or acetate. Large doses of niacin prevented these effects, which therefore were attributed to a limitation of alcohol dehydrogenase through competition for DPN.

Glycolytic Enzymes

Hexokinase.—Hele (149) found the rate of sugar phosphorylation in rat intestine dispersions to vary with both sugar and ATP concentration, with maximum rates for various sugars being obtained at different ATP concentrations. Also, an anomalous effect of glucose concentration was observed, in which intermediate concentrations gave lower rates than either high or low concentrations.

A partially purified hexokinase from particles of calf brain has been studied intensively by Sols & Crane (150). The enzyme was found to attack 16 sugars and sugar derivatives, to be inhibited by 5 related compounds, and to be indifferent to other compounds. For effective reaction with this enzyme,

a substrate must contain a ring structure with hydroxyls in appropriate configuration at carbons 1, 3, 4, and 6. A hydroxyl group at carbon 5 inhibits. The lack of a requirement for a group at carbon 2 is shown by the equivalence of 2-deoxyglucose to glucose. The former compound is recommended for hexokinase studies, as the phosphorylated product does not react with either phosphoglucomutase or phosphohexose isomerase.

Hoare & Kerly (151) found the hexokinase of retina to be activated by Mg^{++} and Mn^{++} and less effectively by Co^{++}. The substrate specificity of this enzyme appears to be similar to that of yeast hexokinase; it attacks only glucosamine and hexoses which have the same configuration on the last four carbon atoms. A hexokinase from *P. putrefaciens* was found by Klein (152) to have high affinity for glucose and glucosamine, low affinity for fructose and mannose. The inhibition of hexokinase of *Clostridium butyricum* by organic phosphate was noted by Gavard (153), while Ivanov & Labanova (154) have found Mg^{++} or Co^{++} to stimulate the enzyme from *Clostridium perfringens*. Sols & Crane (155) report a noncompetitive inhibition of brain hexokinase by sugar derivatives containing a pyranose ring, hydroxyls at carbons 2 and 4, and a phosphate ester at carbon 6.

Further information about the nature of hexokinase in heart muscle was also reported by Sols & Crane (156), who fractionated calf heart homogenates into soluble and insoluble components with kexokinase activity. The particulate enzyme was solubilized by treatment with the detergent Triton X-100 or lipase. The solubilized heart muscle enzyme differs from the original soluble enzyme in its pH activity curve, but both are similar to the particulate brain hexokinase with respect to substrate and competitive inhibitor affinities, and both are inhibited by 6-phosphate compounds more strongly than the brain enzyme.

D-Glucosone, which is not esterified by brain hexokinase, is reported by Eeg-Larsen & Laland (157) to be a potent inhibitor of this enzyme, while D-galactosone is without effect. The inhibition of glucose metabolism in yeast by glucosone (158) may be related to an effect on the same enzyme.

Phosphoglucomutase.—The mechanism of action of this enzyme has been investigated in very direct fashion by Najjar & Pullman (159). Using substrate quantities of the enzyme they have been able to show that the enzyme itself is phosphorylated by glucose-1,6-diphosphate and that the phosphorylated enzyme reacts reversibly with both glucose-1-phosphate and glucose-6-phosphate.

Posternak & Rosselet (160) found that crystalline muscle phosphoglucomutase is capable of reacting with either galactose-1-phosphate or mannose-1-phosphate. As would be expected from the mechanism of action proposed by Najjar & Pullman, the same cofactors are effective with all of the hexose-phosphates. Although the experiments of Klenow (161) indicated that a single enzyme in rabbit skeletal muscle catalyzes the mutation of both hexose and pentose phosphates, this does not appear to be an essential property of mutases. Guarino & Sable (162) have found that phosphoribomutase

activities of yeast and uterine muscle can be distinguished from phosphoglucomutase by their activity in the absence of added cysteine and cations. They reported the preparation of uterine phosphoribomutase free from phosphoglucomutase and capable of attacking deoxyribose-1-phosphate.

Phosphohexose isomerase.—Slein (163) studied the properties of enzymes which reversibly isomerize mannose-6-phosphate (phosphomannose isomerase) and glucose-6-phosphate (phosphoglucose isomerase) to fructose-6-phosphate. It was possible to determine that the fructose phosphate: mannose phosphate ratio at equilibrium is 1.5 at pH 5.5, the optimum for the phosphomannose isomerase since at this pH there is little interference by phosphoglucose isomerase which has a pH optimum at 9. Both enzymes are inhibited slightly by high concentrations of Co^{++}, Mn^{++}, and Mg^{++}, but only the mannose enzyme is inhibited by Ca^{++} or NH_4^+ at pH 7.5. Bruns & Hinsberg (164) measured phosphoglucose isomerase in human serum and found greatly increased levels in certain diseases.

Fructokinase.—This enzyme was purified 250-fold from beef liver by Parks (165). The purification was facilitated by the development of an assay system including phosphocreatine and its transphosphorylase. With this mechanism for preventing the accumulation of the inhibitory ADP[2], greater activity was observed, and the assays became proportional to the amount of enzyme used.

Hexose phosphatases.—Kuhlman (166) has indicated the existence in seminal vesicles and prostate of specific phosphatases for glucose- and fructose-6-phosphates which are localized in different areas from nonspecific acid and alkaline phosphatases.

Phosphofructokinase.—Highly active preparations from rabbit muscle have been obtained in two laboratories. Ling & Byrne (167) found the 160-fold purified enzyme to phosphorylate fructose-6-phosphate and tagatase-6-phosphate but no other sugar phosphates tested. UTP and ITP[2] are almost as active as ATP with purified phosphofructokinase, and the similarities of the Michaelis constants together with the failure of ADP to increase the rates with the other nucleotides are evidence that ITP and UTP are used directly (168). The purified enzyme is activated by metal binding agents and inhibited by *p*-chloromercuribenzoate. Further evidence for the sulfhydryl nature of the enzyme was obtained by Mel'nikova & Neifakh (169) with a 200-fold purified preparation which consisted of an almost homogeneous protein of molecular weight 120,000. This enzyme was estimated to comprise 0.3 to 0.5 per cent of the moist weight of muscle. In addition to free protein thio-groups Mg ions are essential for activity. Phosphofructokinase has a small turnover number and relatively low substrate affinity (K_m for fructose-6-phosphate $= 10^{-3}$ M), and it is suggested by Mel'nikova & Neifakh that it constitutes the factor that limits the rate of glycolysis in muscle.

Hexose diphosphatase.—The Mg ion activated hydrolytic enzyme previously known to remove one of the phosphate groups from hexose diphosphate has been purified extensively from rabbit liver by Pogell & McGilvery

(170). The product was assumed to be fructose-6-phosphate, although frequently high values for labile phosphate were obtained.

Aldolase.—The enzyme from *Trichomonas vaginalis* is activated by Fe^{++} or by Co^{++}, but is inhibited by Zn^{++} (171), whereas the enzyme from *A. niger*, like the well-known yeast enzyme, is activated by Zn^{++} as well as by Fe^{++} and Co^{++} (172). A metal-activated aldolase has also been demonstrated in *C. butyricum* (153). Yeast aldolase has been crystallized as the mercury salt by Warburg & Gawehn (173). The crystalline enzyme is activated by removing mercury with cysteine and adding Zn^{++}.

Mammalian aldolase has been determined in the serum and erythrocytes of many species (174). The serum content of this enzyme was shown to increase markedly in cases of hepatitis (175), as well as in other disease conditions noted earlier. The role of tumor tissue in influencing aldolase levels was shown clearly by Warburg, Gawehn & Lange (176), who found mouse ascites-tumor cells to decrease the aldolase content of serum *in vitro* while fermenting glucose; in the absence of added glucose there was no fermentation, and the aldolase content of the medium increased.

The substrate specificity of muscle aldolase has been reinvestigated. Tung *et al.* (177) have found that aldolase activity is not restricted to compounds with the *trans* configuration at carbons 3 and 4, but will also cleave tagatose-1,6-diphosphate between the 3 and 4 carbons, which bear *cis*-hydroxyls. The condensation of dihydroxyacetone phosphate with both D and L-glyceraldehyde has been shown to be reversible, the affinity for fructose-1,6-diphosphate has been found to be much greater than the affinity for fructose-1-phosphate, and various hexose-6-phosphates were found to give no reaction with aldolase. Although aldolase was found not to possess specificity for a *trans* configuration, Byrne & Lardy (178) reported that D-xylulose is formed when dihydroxyacetone phosphate is condensed with either glycolaldehyde or phosphoglycolaldehyde, confirming the preference for the *trans* structure.

Leuthardt & Wolf (179) have confirmed the conclusion of Hers & Kusaka (180) with respect to differences in liver and muscle aldolase. Fructose-1-phosphate is split rapidly only by the liver enzyme, whereas fructose-1,6-diphosphate is attacked by both. Leuthardt & Wolf have also found the liver enzyme to be much more active than the muscle enzyme in catalyzing reactions with several nonphosphorylated aldehydes, including glycolaldehyde and glyceraldehyde.

Additional studies on the enzyme, distinct from aldolase, which carries out an aldol condensation between dihydroxyacetone phosphate and formaldehyde have been reported by Charalampous (181). The enzyme was purified from rat liver to give a ratio of activities compared with aldolase of 6, whereas the ratio in crude extracts was 0.2. The pH optima also differ, being 7.2 for the formaldehyde enzyme and 9.0 for aldolase. The product of the condensation was identified as erythrulose-1-phosphate.

Triose phosphate dehydrogenase.—For several years there has been general

acceptance of the conclusion that there is no interaction between the aldehyde and inorganic phosphate prior to oxidation of the substrate. The opposing argument has been vigorously supported by Warburg and collaborators (182), who reported briefly on experiments showing the rate of nonenzymatic interaction between both DPN and glyceraldehyde and between DPNH[3] and glycolaldehyde to be increased with increasing phosphate concentration. Unfortunately, these experiments cannot be evaluated without additional information. In particular, it is desirable to know that the optical density change at 340 mμ really represents pyridine nucleotide reduction or oxidation and that the effects observed are specific for phosphate. Warburg *et al.* also criticize the experiments of numerous other workers who have used stoichiometric amounts of enzyme on the ground that reaction rates were not studied; they state that with glyceraldehyde as the substrate they find a stimulation in the rate (measured in the first 3 sec.) by inorganic phosphate. Again, it is unfortunate that more information was not presented to establish that the effect of phosphate is on the initial rate of DPN reduction, or that phosphate permits a rapid displacement of an equilibrium with the more extensive reaction in the presence of phosphate appearing as a more rapid rate. The use of acetaldehyde as a substrate, which would decrease the rate further, should permit this point to be settled.

Evidence that the reaction of triosephosphate dehydrogenase with phosphate comes subsequent to the oxidation of aldehyde was offered by other workers. Harting & Velick (183, 184) reported further on their experiments with acetaldehyde and other slowly oxidized substrates. Acetaldehyde, propionaldehyde, and butyraldehyde are oxidized at only 2 to 5 per cent of the rate of glyceraldehyde. In the presence of inorganic phosphate the corresponding acyl phosphates accumulate, but in the absence of both phosphate and arsenate the oxidation proceeds at only a slightly lower rate; presumably the free acids are formed. Both Harting & Velick (184) and Oesper (185) have studied the ability of triosephosphate dehydrogenase to react with acyl phosphates. Oesper measured the exchange between the acyl phosphate of 1,3-diphosphoglyceric acid, using inorganic P^{32} phosphate, and found the exchange to proceed much more rapidly than could be accounted for by reduction and reoxidation. Similarly, Harting & Velick found the acetyl group of acetyl phosphate to be transferred to inorganic phosphate, CoA,[2] or glutathione in the absence of reducing power. Both groups also measured hydrolysis in the presence of arsenate. For all of these reactions DPN is necessary. The conclusion was reached in agreement with previous hypotheses (186, 187, 188) that the initial oxidation of aldehyde results in the formation of acyl-enzyme compounds which react secondarily with any of a number of acyl acceptors, including inorganic phosphate. However, the role of DPN in reactions not involving oxidations remains unclear.

Krimsky & Racker (189) have presented new evidence to strengthen their contention that glutathione is an active participant in the oxidation of aldehydes. They have now been able to obtain as much as 1.6 equivalents

of glutathione liberated per mole of enzyme by tryptic digestion. Evidence for the participation of glutathione in the exchange reaction catalyzed by triosephosphate dehydrogenase was obtained by permitting C^{14}-labeled acetyl phosphate to react with the enzyme, which was then digested with trypsin. Chromatography of the digest on Dowex-50 and on paper showed the existence of a labeled compound migrating with acetylglutathione.

Additional information about the binding of DPN to the dehydrogenase has come from studies on the availability of bound DPN to other enzymes. Mahler & Elowe (190) found that only one of the two molecules of DPNH bound to muscle triosephosphate dehydrogenase is oxidized by DPN cytochrome-*c* reductase. The rate of the oxidation is equal to that with free DPNH. Astrachan (191) found that DPN bound to enzyme inactivated by oxidation of sulfhydryl groups is susceptible to certain enzymes which do not attack the DPN of active enzyme. A phosphodiesterase and DPN-kinase attack bound DPN, while diphosphopyridine nucleotidase attacks bound DPN only outside the pH range 7.4 to 8.0. Bound DPN becomes a substrate for both a deaminase and certain dehydrogenases on reduction.

The nature of the destruction of DPNH by triosephosphate dehydrogenase has been further studied by Rafter, Chaykin & Krebs (192). They determined that the enzyme catalyzes a reaction at pH 5 to give a product with the spectrum produced nonenzymatically at lower pH values with a peak at 280 to 290 mμ. The enzymatic reaction requires polyvalent anions and is inhibited by DPN. The modification of DPNH is catalyzed by both yeast and muscle triosephosphate dehydrogenase, but by neither yeast alcohol dehydrogenase nor heart lactic dehydrogenase.

In plants the existence of several enzymes capable of oxidizing phosphoglyceraldehyde has been shown. In addition to the counterpart of the phosphate-requiring, DPN enzyme of animal and microbial organisms and the previously described phosphate-TPN[2] enzyme (193), Arnon, Rosenberg & Whatley (194) describe the preparation of a protein fraction from green leaves which uses TPN to oxidize phosphoglyceraldehyde to 3-phosphoglyceric acid without the participation of inorganic phosphate.

Enolase.—The formation of complexes of Mg^{++}, Mn^{++}, and Zn^{++} with 2-phosphoglyceric acid have been studied by Malmström (195), who found that 1:1 complexes were formed. The dissociation constant for the Zn^{++} complex is a much larger number than the corresponding Michaelis constant. It was concluded that metal activation of enolase represents combination of the metal with the enzyme and not formation of a metal-substrate complex.

Lactic dehydrogenase.—Hill & Mills (196) report that lactic dehydrogenase is lacking in *Bacterium tularense*, *Brucella abortus*, *Agrobacterium tumefaciens*, and *Mycobacterium phlei*, all of which oxidize glucose but do not glycolyze. Sonic extracts of these organisms were found to produce lactic acid when supplemented with heart muscle lactic dehydrogenase. The bacteria are capable of oxidizing lactic acid but not with DPN. The lactic oxidase of *B. tularense*

has been shown to be free from cytochrome-*b*, which differentiates it from a similar yeast enzyme. Another lactic enzyme was purified 200-fold from sonic extracts of dried *Proteus vulgaris* by Moyed & O'Kane (197). The enzyme reduces 2,6-dichlorophenol indophenol, but is inert with respect to pyridine nucleotides. No cytochromes were found in the purified enzyme nor was there evidence for a flavin component. A similar enzyme was obtained by Szulmajster *et al.* (198) from particles of *Escherichia coli*. Particles were obtained by sonic disintegration and enzymatic digestion, and the enzyme was solubilized with butanol. Dichlorophenol indophenol reduction, in this case, also was independent of pyridine nucleotides, and neither flavin nor heme was detected. The enzyme appears to be specific for L-lactate.

An oxidative decarboxylation of lactate to acetate and CO_2 was reported by Sutton (199) to be catalyzed by an enzyme from *Mycobacterium phlei*. Previously Edson (200) had purified an enzyme from this organism and found it to reduce methylene blue anaerobically and to accumulate pyruvate, whereas aerobically acetate and CO_2 were formed. Edson presented evidence that the enzyme involved is a flavoprotein and that acetate is formed by a nonenzymatic reaction of pyruvate with H_2O_2. Sutton used dialyzed extracts and found that neotetrazolium was not reduced. He also found that such preparations did not oxidize pyruvate and were not influenced by catalase which would interfere with peroxidation of pyruvate. It was suggested by Sutton that two pathways for lactate degradation exist in *M. phlei*. If so, five enzymatic types will have been found to oxidize lactate: a pyridine nucleotide type, a flavoprotein, cytochrome-*b*, an oxidase without known cofactors, and the proposed oxidative decarboxylation type. The lactic dehydrogenase of yeast was crystallized by Morton & Appleby (201) and found to contain flavin in addition to cytochrome-*b*.

Crystalline lactic dehydrogenase from heart muscle was found by Nielands (202) to react very slowly with *p*-chloromercuribenzoate. The treated inactive enzyme could be reactivated very rapidly by cysteine. It was concluded that lactic dehydrogenase, and DPN enzymes in general, require -SH groups for activity. However, since neither iodosobenzoate, iodoacetate, nor N-ethylmaleimide inactivated the enzyme, the conclusion that a sulfhydryl group is used for binding DPN must be considered tentative. It was claimed by Etling (203) that certain hypnotic drugs had no effect on any of the enzymes of glycolysis except lactic dehydrogenase. However, since the assay for lactic dehydrogenase involved dye reduction, it cannot be ascertained whether the dehydrogenase or a diaphorase was inhibited.

Alcohol dehydrogenase.—Hayes & Velick (204) have found that crystalline alcohol dehydrogenase of yeast has a molecular weight of 150,000 and 4 equivalent binding sites for DPN or DPNH. DPNH is bound about 5 to 10 times more tightly than DPN, as determined by activity measurements and by an ultracentrifugation method. At high enzyme concentrations the apparent equilibrium of the reaction is shifted because of binding of DPNH to the enzyme. The equilibrium constant obtained at lower enzyme concentra-

tions agrees with the Haldane relationship between equilibrium constant, maximum velocities, and Michaelis constants. This relationship was found by Alberty (205) not to hold for the data of Theorell & Bonnichsen (206) with liver alcohol dehydrogenase. Another difference between the yeast and liver enzymes reported by Hayes & Velick is the lack of spectral shift on combination of DPNH with the yeast enzyme.

DeMoss (207) has found that *L. mesenteroides* contains an alcohol dehydrogenase specific for TPN in addition to a DPN enzyme.

Preparation of glycolytic enzymes.—Rabbit muscle has long been a favorite source for glycolytic enzymes, many of which have been isolated as homogeneous crystals. The occurrence of these enzymes in high concentration has permitted the development of a fractional crystallization procedure which yields in one process five crystalline enzymes: aldolase, triosephosphate dehydrogenase, glycerophosphate dehydrogenase, lactic dehydrogenase and pyruvate kinase (208).

Compounds Related to Glycolytic Metabolites

Methylglyoxal.—The role of methylglyoxal in intermediary metabolism remains obscure. It was reported by Salem (209) that methylglyoxal is excreted by thiamine-deficient rats. Such animals have less glyoxalase in their livers than normal and accumulate methylglyoxal when hexose diphosphate is added *in vitro*. These changes are reversed by prior feeding of thiamine. Meyer (210) has added to the evidence excluding methylglyoxal from glycolysis. His experiments showed that spontaneous methylglyoxal formation from glyceraldehyde is not accelerated by muscle extracts, and that dephosphorylation of triose phosphate proceeds faster than methylglyoxal formation.

Propanediol.—Rudney (211) has claimed that propanediol phosphate comprises 1 to 2 per cent of the acid soluble phosphate of liver. These figures were obtained by an isotope dilution technique, which unfortunately cannot be accepted literally, since the compound isolated was not analytically pure. It is probable, however, that propanediol and its phosphate do occur in liver since propanediol is metabolized actively, contributing to both the formate pool and lactate (212). In yeast, also propanediol is reported by Huggins & Miller (213) to be phosphorylated and also converted to lactate and other unidentified products. Propanediol phosphate, acetol, and lactate were all reported to form propanediol.

Glycols.—A family of DPN-requiring dehydrogenases attacking glycols was indicated by Goldschmidt & Krampitz (214) to be present in *Acetobacter suboxydans*. 2,3-Butylene glycol, propylene glycol, and ethylene glycol are all oxidized to the corresponding ketols, but since the relative rates vary, it is suggested that three enzymes are present.

Glycerol.—The distribution of radioactive glycerol in the intact rat was followed by Gidez & Karnosvky (215) who found rapid incorporation into

blood glucose and liver glycogen. The specific activity of triglyceride glycerol reached three times that of the phosphatides.

In recent years several examples of asymmetric reactions of symmetrical compounds have been reported in support of the Ogsten hypothesis. Schambye, Wood & Popják (216) isolated glycerol-1-C^{14} from goats fed carboxyl-labeled acetate. This glycerol was found to give glycogen labeled predominantly in the 3,4-positions, with substantially greater activity in the 3-position. In a simultaneous publication, Swick & Nakao (217) reported isolation of glycerol from 3,4-labeled glucose. When this was fed to rats, 91 per cent of the radioactivity of the glycerol was returned to the 3,4 positions.

Magasanik *et al.* (218) found that two pathways for glycerol oxidation exist in different strains of *Aerobacter aerogenes*. A capsulated strain oxidizes glycerol to dihydroxyacetone, whereas an acapsulated strain first forms L-α-glycerophosphate, which is then oxidized. In *A. suboxydans* it was shown by Hauge, King & Cheldelin (219) that glycerol oxidation depends upon prior phosphorylation which apparently produces α-glycerophosphate since this compound is oxidized rapidly, whereas the β-phosphate is not oxidized. In this organism, dihydroxyacetone is also oxidized following phosphorylation. Wiame, Bourgeois & Lambion (220) have found a dehydrogenase in *B. subtilus* which uses DPN to oxidize glycerol to dihydroxyacetone. In this organism apparently only the subsequent oxidation of dihydroxyacetone requires the presence of phosphate in the molecule. A kinase, triokinase, was found in a strain which can oxidize dihydroxyacetone, but was lacking in a strain which accumulates this compound.

OTHER MECHANISMS OF CARBOHYDRATE METABOLISM

The Pentose Phosphate Pathway

Recent studies on the role of pentose and heptulose phosphates in carbohydrate metabolism have led to the elaboration of a cyclic process in which these esters occupy a central role. In this section recent developments in this field are discussed and formulated into a tentative scheme, which may provide a basis for experiments designed to test the biological role of these reactions.

Glucose-6-phosphate and 6-phosphogluconate oxidation.—New experiments have confirmed the presence of glucose-6-phosphate dehydrogenase (*Zwischenferment*) and 6-phosphogluconic dehydrogenase in a number of additional types of biological material, including species of Pseudomonas (221, 222), Leuconostoc (223), Azotobacter (224), Bacillus (225), and Achromobacter (131), and green leaves of plants (226, 227), as well as in a variety of animal tissues (228, 229). The oxidation of glucose-6-phosphate was shown to be reversible in the sense that the first oxidation product, 6-phosphoglucono-δ-lactone, is reducible by $TPNH^2$ in the presence of the dehydrogenase (230). At neutral pH hydrolysis of the lactone with the formation of the ionized carboxyl group shifts the over-all equilibrium in the direction of

6-pho phogluconate formation. The following sequence of reactions is involve in glucose-6-phosphate oxidation:

glucop anose-6-phosphate + $TPN^+ \rightleftarrows$ 6-phosphoglucono-δ-lactone + TPNH + H^+ 1.
6-phos hoglucono-δ-lactone + $OH^- \rightleftarrows$ 6-phosphogluconate$^-$ 2.
6-phos hogluconate$^-$ + $TPN^+ \rightleftarrows$ ribulose-5-phosphate + CO_2 + TPNH 3.

In the presence of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehyc ogenase reaction 2 becomes limiting, since the spontaneous rate of hydro sis of the lactone ring is relatively slow. For this oxidation pathway to hav physiological significance it was necessary to postulate the existence of an nzyme which catalyzes the hydrolysis of the lactone. Such an enzyme has n w been discovered by Brodie & Lipmann (231) in extracts of *Azotobacter inlandii* and in animal tissues. This enzyme catalyzes the hydrolysis of δ-gl conolactone, as well as the 6-phosphoderivative and is undoubtedly involv d in glucose-6-phosphate oxidation in the intact cell. No *in vivo* or *in vitr* evidence for the fixation of CO_2 into hexose by this mechanism is yet availa le, although all of the steps now appear to be reversible.

Th product of 6-phosphogluconate oxidation by extracts of *L. mesenteroide* (232) has been shown to be ribulose phosphate, just as in animal tissue or yeast. With *Azotobacter* extracts Mortenson & Wilson (224) have also ob ained pentose phosphate as a reaction product, although no pyridine nucleo de requirement could be demonstrated for this step. Phosphoribose isomer se, which catalyzes the conversion of ribulose phosphate to ribose phosph ate has been purified about 400-fold from alfalfa by Axelrod & Jang (233). At 37°C. and pH 7.0 $\Delta F = -0.7$ kcal. for the reaction ribulose-5-phosph ate$\rightleftarrows$ribose-5-phosphate. The heat of the reaction was 3.1 kcal., from w hich the entropy change was estimated to be 12.1 kcal. This large entrop change was attributed to the stabilizing effect of the hemi-acetal ring. T e isomerase resembles other enzymes of this type in exhibiting an unusua ly high activity; the turnover number under the standard assay condit ns was over 200,000. This enzyme was inactive with a number of other s bstrates tested and was inhibited by phosphoribonic acid.

Tra sketolase and the formation of sedoheptulose-7-phosphate.—The mechanism of sedoheptulose phosphate formation from pentose phosphate has been f rther clarified and the reaction shown to be reversible. Horecker, Smyrni tis & Klenow (234) have described procedures for the purification of tran ketolase from extracts of rat liver and from spinach. The prosthetic group of both enzymes was identified as thiamine pyrophosphate, and this has als been established by Racker, de la Haba & Leder (235) for the crystalline yeast enzyme. With the purified liver and spinach enzyme preparations, which contain detectable amounts of phosphoribose isomerase, the reaction leads reversibly to the formation of sedoheptulose-7-phosphate:

2 pento e phosphate$\rightleftarrows$sedoheptulose-7-phosphate+glyceraldehyde-3-phosphate 4.

The equilibrium for reaction 4 seems to favor the formation of pentose phosphates. However, about 80 per cent of the pentose ester added (either ribu-

lose-5-phosphate or ribose-5-phosphate) is converted to the heptulose ester by the liver enzyme preparations when crystalline muscle aldolase is added. The mechanism of the aldolase effect is not known, since the spinach preparations which are free of aldolase form the same amount of heptulose without this addition and appear to contain an additional factor that produces the same result.

With the crystalline yeast preparation and with ribulose-5-phosphate or hydroxypyruvate as substrates, Racker, de la Haba & Leder (235) have found a requirement for an acceptor aldehyde, and this has led them to postulate that a bound form of glycolaldehyde, designated as "active glycolaldehyde," is an intermediate in the reaction and to suggest the name transketolase for the enzyme. A number of substances have now been shown to serve as "active glycolaldehyde" donors or acceptors (234 to 237). These are summarized as follows: (*a*) "Active Glycolaldehyde" Donors: Ribulose-5-phosphate, L-erythrulose, sedoheptulose-7-phosphate, hydroxypyruvic acid, and fructose-6-phosphate. (*b*) "Active Glycolaldehyde" Acceptors: D-Glyceraldehyde-3-phosphate, L-glyceraldehyde-3-phosphate, ribose-5-phosphate, glycolaldehyde, erythrose-4-phosphate, and D,L-glyceraldehyde.

Presumably any compound in the first group will provide "active glycolaldehyde," which can condense with any acceptor in the second group. Of the many possible combinations of reactions catalyzed by transketolase, the following appear to be of particular significance and will be discussed again later:

$$\text{ribose-5-phosphate} + \text{ribulose-5-phosphate} \rightleftarrows \text{sedoheptulose-7-phosphate} + \text{glyceraldehyde-3-phosphate} \quad 5.$$

$$\text{fructose-6-phosphate} + \text{glyceraldehyde-3-phosphate} \rightleftarrows \text{ribulose-5-phosphate} + \text{erythrose-4-phosphate} \quad 6.$$

The formation of heptulose phosphate is now reported to occur with crystalline yeast transketolase (238) as well as with the liver and spinach preparations.

On the basis of the substrate and acceptor requirements and by analogy with the formation of acetoin, the mechanism shown in Figure 1 has been proposed (238, 239) for the formation of sedoheptulose-7-phosphate from pentose phosphate.

Presumably, the ThPP[2] moiety participates in the formation of the glycolaldehyde-enzyme complex, but the nature of this linkage is not known, nor is there any direct evidence for the "active glycolaldehyde" intermediate. ThPP itself does not appear to function as an acceptor (240).

Whether the *cis* or *trans* configuration arises in the condensation with "active glycolaldehyde" appears to be determined by the thermodynamic equilibrium, rather than any directing action of the enzyme. D-Xylulose-5-phosphate will react with transketolase at about the same rate as D-ribulose-5-phosphate and is probably produced together with ribulose phosphate when D-glyceraldehyde-3-phosphate serves as the acceptor (241).

Crystalline muscle aldolase or a pea aldolase preparation catalyzes the

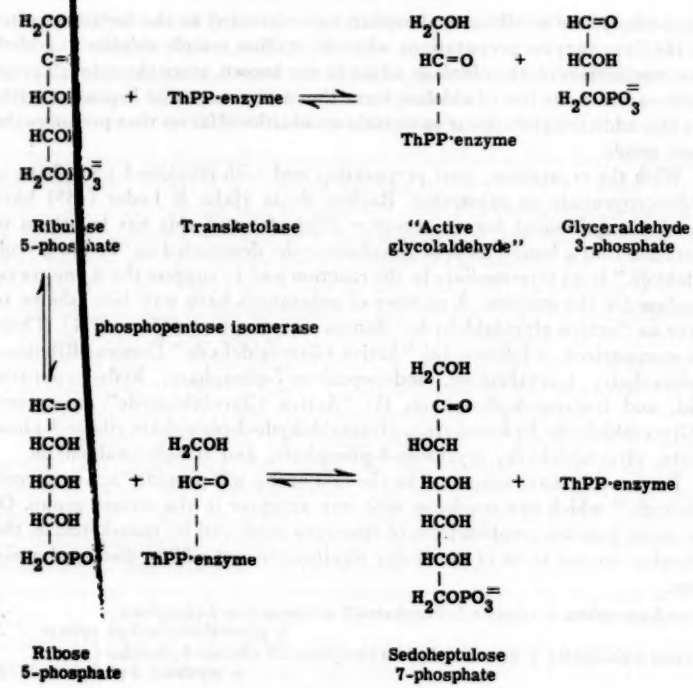


FIG. 1. Proposed mechanism for formation of sedoheptulose-7-phosphate from pentose phosphate.

formation of sedoheptulose-1-phosphate from dihydroxyacetone phosphate and D-erythrose (234, 242), but this ester is not an intermediate in sedoheptulose-7-phosphate formation from pentose phosphate in the reaction catalyzed by transketolase. The same is true for L-erythrulose, which will act as a source of "active glycolaldehyde" and which will therefore give rise to both pentose phosphate and heptulose phosphate in the presence of purified spinach transketolase and D-glyceraldehyde-3-phosphate. However, experiments with C^{14}-labeled pentose phosphate do not support a mechanism in which L-erythrulose is an intermediate (234, 243, 244).

The preparation of sedoheptulose-7-phosphate has been accomplished by the action of spinach or liver transketolase on ribose-5-phosphate (234). The product is separated from residual pentose esters by chromatography on Dowex 1 formate columns and isolated as the barium salt. The product is about 75 per cent pure and is free of other phosphate esters.

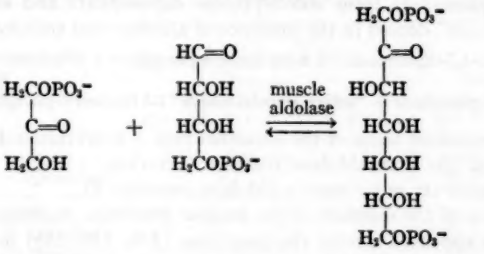
Transaldolase: The conversion of sedoheptulose-7-phosphate to fructose-6-phosphate.—This enzyme, which catalyzes the reaction:

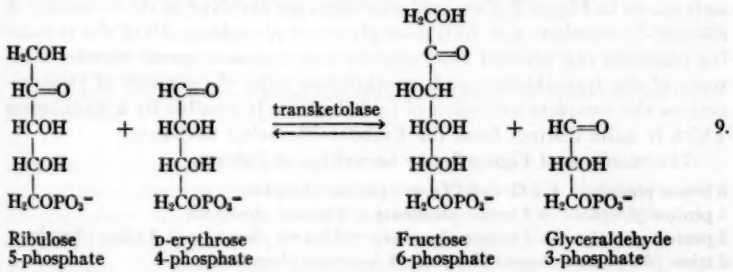
Sedoheptulose-7-phosphate + triose phosphate $\rightleftarrows$ fructose-6-phosphate + erythrose-4-phosphate 7.

has now been obtained from yeast in highly purified form (245). The reaction appears to be reversible, with an equilibrium constant which is close to 1.0. The hexose ester formed has been identified as fructose-6-phosphate in confirmation of an earlier observation of Glock (246) who found fructose-6-phosphate to be the first product formed from pentose phosphate with liver extracts. The tetrose ester which was detected on paper chromatograms will be discussed in the next section. Bergmann, Littauer & Volcani (247) have obtained evidence for transaldolase in *E. coli*. In extracts of this organism the utilization of sedophetulose-7-phosphate is greatly accelerated by the addition of fructose-1,6-diphosphate.

Sato *et al.* (248) have isolated an organism by enrichment culture which is capable of growth on sedoheptulose. The oxidation of this substrate by acetone-dried cells requires phosphate or ATP, suggesting that a phosphate ester is an intermediate.

The metabolism of D-*erythrose-4-phosphate.*—The identification of this product, which has yet to be isolated, is based on two reactions which have been observed in solutions in which it is generated:





Reaction 8 represents a condensation catalyzed by crystalline muscle aldolase (249) in the presence of fructose diphosphate as a source of dihydroxyacetone phosphate. The product has been isolated by ion exchange chromatography and identified as sedoheptulose-1,7-diphosphate (250), which could only have arisen from an aldotetrose with the D-erythrose configuration. Since sedoheptulose diphosphate is rapidly cleaved by aldolase, which has a high affinity for this substrate, it may have physiological importance, perhaps as a reservoir for the very reactive tetrose ester. Earlier suggestions (251, 252) that a sedoheptulose derivative serves as a precursor of shikimic acid in aromatic ring synthesis have received experimental support from the work of Davis and Sprinson and their collaborators, who found that extracts of *E. coli* mutants utilize sedoheptulose diphosphate efficiently for the formation of dehydroquinic acid, which in turn is converted to shikimic acid. [See Kalan & Srinivasan (253).]

Experiments carried out with ribose-5-phosphate-1-C^{14} and -2,3-C^{14} with enzyme preparations from liver (243) and from pea leaves (244) have been interpreted in terms of a condensation of "active glycolaldehyde" with D-erythrose-4-phosphate (see reaction 9). More direct evidence for such a reaction has been obtained by Racker, de la Haba & Leder (236) who find fructose-6-phosphate to act as an "active glycolaldehyde" donor with transketolase. Horecker & Smyrniotis (254) have observed the formation of fructose-6-phosphate from sedoheptulose diphosphate and several "active glycolaldehyde" donors in the presence of aldolase and transketolase:

Sedoheptulose-1,7-diphosphate $\rightleftarrows$ D-erythrose-4-phosphate + dihydroxyacetone phosphate 10.

D-erythrose-4-phosphate + "active glycolaldehyde" $\rightleftarrows$ fructose-6-phosphate 11

These observations support the identification of D-erythrose-4-phosphate as a product of the transaldolase reaction (reaction 7) and of sedoheptulose diphosphate cleavage by muscle aldolase (reaction 8).

Summary of the reactions of the pentose phosphate pathway.—Several reviews have appeared during the past year (238, 239, 255) in which recent developments in this field have been summarized. The reactions discussed in the preceding sections can be formulated into a cyclic mechanism, such as is shown in Figure 2. Two oxidative steps are involved in the formation of ribulose-5-phosphate and CO_2 from glucose-6-phosphate. All of the remaining reactions can proceed anaerobically and represent group transfer reactions of the transaldolase and transketolase type. As a result of these reactions the complete oxidation of carbohydrate is possible by a mechanism which is quite distinct from the Embden-Meyerhof pathway.

The reactions of Figure 2 may be written as follows:

6 hexose phosphate + 3 O_2 → 6 CO_2 + 6 pentose phosphate
4 pentose phosphate → 2 hexose phosphate + 2 tetrose phosphate
2 pentose phosphate + 2 tetrose phosphate → 2 hexose phosphate + 2 triose phosphate
2 triose phosphate → hexose phosphate + inorganic phosphate

Sum: hexose phosphate + 3 O_2 → 6 CO_2 + inorganic phosphate

The role of the pentose phosphate pathway in animal tissues.—Apart from a possible function in carbohydrate oxidation, the reactions outlined in Figure 2 provide two essentially distinct mechanisms for the biosynthesis of ribose phosphate. These are (*a*) the oxidative decarboxylation of 6-phosphogluconate and (*b*) a reversal of the transketolase-transaldolase sequence. Racker *et al.* (236) have demonstrated the formation of ribulose-5-phosphate from fructose-6-phosphate (reaction 6, p. 231). This product would contain carbon atoms 1 and 2 of the hexose in positions 1 and 2 of pentose. Two more molecules of pentose phosphate would arise by a reversal of the anaerobic reactions leading to erythrose-4-phosphate formation. (For an elaboration of this scheme see p. 233). Calculations made from the results reported by Bernstein (256) indicate that in the intact chick the second pathway predominates and that little, if any, pentose phosphate is formed by the loss of carbon atom 1 of hexose phosphate. Bernstein compared the C^{14} distribution in ribose isolated from visceral ribonucleic acid with that found in the glucose of liver glycogen and concluded that neither the oxidation of hexose nor the decarboxylation of uronic acid could account for the observed labeling in pentose phosphate. However, the data shown in Table I are in excellent

TABLE I

RELATIVE C^{14} DISTRIBUTION IN RIBOSE AND GLYCOGEN OF CHICKS FED LABELED COMPOUNDS*

Substrate		C^{14} Distribution in										
		Glucose						Ribose				
		C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5
$CH_3C^{14}OONa$	Found	2	1	100	100	1	2	10	16	100	3	0
	Calculated†							35	34	100	1	2
$C^{14}H_2NH_2COOH$	Found	100	133	16	16	133	100	79	98	27	124	100
	Calculated†							72	94	16	133	100
$HC^{14}OOH$	Found	100	41	90	90	41	100	79	51	98	45	100
	Calculated†							97	57	90	41	100
$HC^{14}OOH$	Found	100	37	20	20	37	100	69	31	26	42	100
	Calculated†							73	31	20	37	100

* Taken from the data of Bernstein (256).

† Calculated from the observed isotope distribution in glucose and the transketolase and transaldolase sequence.

agreement with values predicted by the transketolase-transaldolase pathway. Poorest agreement is given in the experiment with carboxyl-labeled acetate, where the discrepancy can be attributed to a higher labeling in C-1 of triose phosphate than in C-3 and C-4 of hexose phosphate. It is of interest to compare this result with that obtained with photosynthesizing algae (see p. 251).

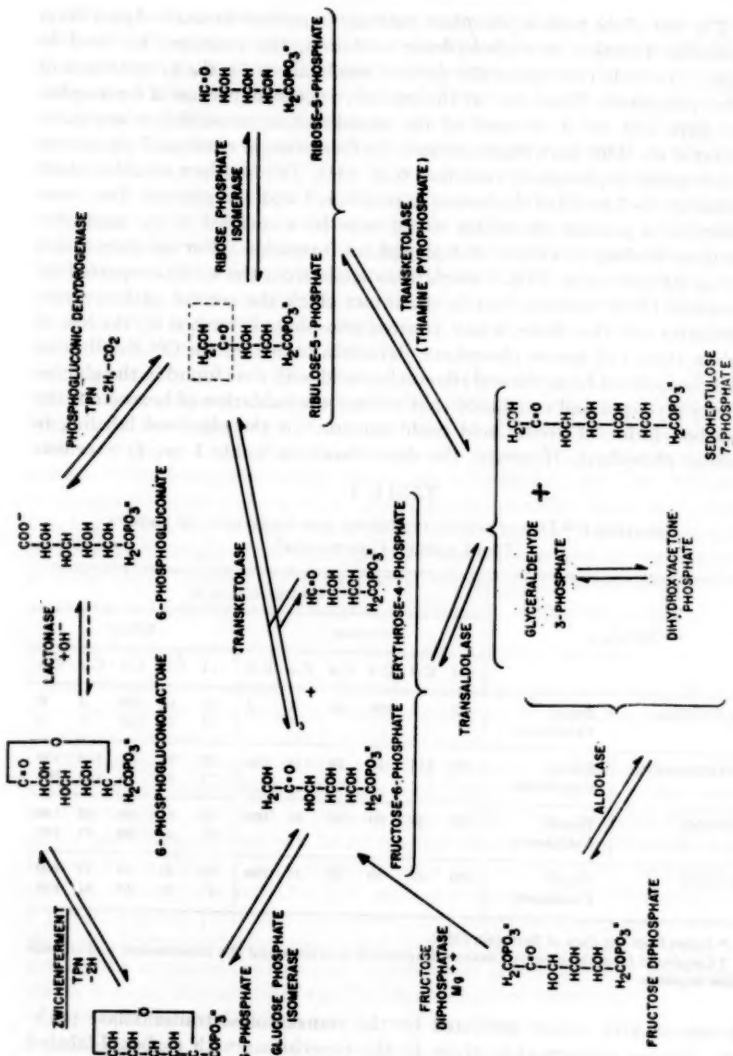


FIG. 2. Glucose-6-phosphate metabolism by the pentose phosphate pathway.

Plaut & Broberg (257) have degraded riboflavin formed from C-1 labeled glucose and found that isotope appears in the C-1 and C-5 positions of the ribityl group. Reaction 6 could account for the incorporation of isotope into C-1 by way of a pentose phosphate precursor.

Numerous efforts have been made, employing labeled substrates, to evaluate the quantitative significance of the pentose phosphate pathway in the oxidation of carbohydrate. With the aid of glucose-1-C^{14} and glucose-6-C^{14} Bloom & Stetten (258) have continued their investigation of the role of the oxidative pathway in glucose catabolism. In confirmation of their earlier results (259) they found that kidney slices and diaphragm sections form CO_2 from C-1 and C-6 of glucose at nearly equal rates, as would be expected from the Embden-Meyerhof mechanism, whereas with liver slices, carbon 1 is converted to CO_2 at three times the rate observed with carbon atom 6.

With respect to the formation of CO_2 from labeled glucose with liver slices, the earlier data of Bloom *et al.* (259) have been re-evaluated by Katz *et al.* (260), who conclude that 80 to 90 per cent of the CO_2 from glucose arises by a glycolytic mechanism (via lactic or pyruvic acid). However, it is assumed that in the pentose phosphate pathway only carbon atom 1 of glucose contributes to the CO_2. The validity of this assumption is open to some doubt, particularly since active mechanisms are known for generating hexose phosphate from pentose phosphate. In view of the complicated pattern of carbohydrate interconversions which are possible by operation of the transketolase-transaldolase sequence, it is necessary to interpret the results of CO_2 evolution from labeled precursors with considerable caution. However, the ratio of CO_2 formed from glucose-1-C^{14} and glucose-6-C^{14} is useful as a qualitative index of the occurrence of this pathway. The effect of physiological conditions on this ratio in rat liver slices has been studied by Agranoff *et al.* (261), who found that regenerating and neoplastic liver slices showed a shift toward the Embden-Meyerhof mechanism, while in slices of maternal liver during pregnancy the contribution of the pentose phosphate pathway was increased. A similar effect of physiological conditions was noted by Glock & McLean (229), who found a large increase in the levels of *Zwischenferment* and 6-phosphogluconic dehydrogenase in the mammary gland of the rat during lactation and a rapid return to normal during involution. Glock & McLean (228) have found high levels of these enzymes in adrenal cortex, lymph, and embryonic tissues and have confirmed the low levels previously reported for muscle. A significant observation is the rapid conversion of pentose phosphate to hexose phosphate by muscle extracts, indicating that the anaerobic reactions of the cycle involving transketolase and transaldolase are present in muscle and suggesting that these enzymes may function in processes which are not necessarily oxidative. A variety of tumors tested showed normal levels of these enzymes. The inhibitory effect of hypnotics on the respiration of brain homogenates has been attributed by Etling (262) to an effect of these agents on the oxidation of glucose-6-phosphate by *Zwischenferment.*

FIG. 2. Glucose-6-phosphate metabolism by the pentose phosphate pathway.

Kelly *et al.* (263) have also found high glucose-6-phosphate dehydrogenase activity in adrenal tissue together with evidence for the conversion of pentose phosphate to hexose phosphate by way of heptulose phosphate. In adrenal homogenates the oxidation of hexose followed a nonglycolytic, iodoacetate-insensitive pathway.

Red cells from vitamin B_{12}-deficient rats show an impaired ability to form ribose *in vitro*, which may be related to their reduced oxygen consumption and low blood glutathione levels. Ling & Chow (264) found that both ribose synthesis and oxygen consumption were restored by the addition of reduced glutathione and suggested that ribose formation may be an important pathway of glucose utilization in mammalian erythrocytes.

The pentose phosphate pathway in microorganisms and higher plants.—A number of investigations have been carried out to evaluate the role of these reactions in yeast and other microorganisms. Lanning & Cohen (265) examined the ribose formed in *E. coli* grown on glucose-1-C^{14} as the sole carbon source, found it to be about 30 per cent as active as the initial glucose and concluded that the major pathway of ribose synthesis involves the loss of C-1 of the hexose. In similar experiments with *Torula utilis*, Sowden *et al.* (266) found only traces of radioactivity in the nucleic acid ribose. David & Renault (267) employed glucose-2-C^{14} in an effort to avoid the negative type of result. Ribose isolated from the nucleic acid of *T. utilis* grown on this substrate was slightly more radioactive than the original glucose, and it was established that the activity resided in the first three carbon atoms. While this result can be interpreted in terms of either C-1 oxidation or "active glycolaldehyde" transfer from hexose phosphate to triose phosphate, when it is analyzed in conjunction with the data of Sowden *et al.*, it appears that pentose synthesis in *T. utilis* occurs almost exclusively by the oxidation of 6-phosphogluconate. Further evidence for the occurrence of a C-1 oxidation in yeast has been obtained by Beevers & Gibbs (268) with resting cell suspensions of *Saccharomyces cerevesiae*. The rate of CO_2 production from C-1 and from C-6 labeled glucose ranged from 2.0 with cells grown with vigorous aeration to 5.2 with cells grown without aeration. No attempt was made to assign values for the extent of participation of the various pathways.

A somewhat different approach has been employed by Lewis *et al.* (269) based on the assumption that pyruvate (or lactate) arises equally from glucose carbons 1 to 3 and 4 to 6 by the Embden-Meyerhof pathway, while only carbon atoms 4 to 6 would contribute to these substances when glucose is metabolized by the pentose pathway. The results of analysis of acetate and acetoacetate formed from glucose-1-C^{14} and glucose even-C^{14} indicated that glucose catabolism by the Embden-Meyerhof pathway accounted for 12 to 36 per cent of the total triose units formed in liver slices or homogenates, while in kidney and hepatoma the values were closed to 100 per cent. In two strains of yeast under aerobic conditions and in *E. coli* and *P. chrysogenum* the Embden-Meyerhof pathway accounted for 50 to 80 per cent of glucose breakdown. However, the basic assumption must be re-evaluated to consider the

effect of pentose synthesis by the fructose phosphate-triose phosphate condensation.

Beevers & Gibbs (270, 271) have studied the metabolism of glucose in higher plants and have concluded that, while in meristematic root tissue the glycolytic mechanism predominates, carrot root slices and leaf and stem tissue contain a mechanism for the preferential conversion of C-1 of glucose to CO_2. A number of carbohydrate intermediates are oxidized by pea leaf extracts (226) including fructose-6-phosphate and glucose-6-phosphate with TPN as the coenzyme. This coenzyme is also needed for the oxidation of fructose diphosphate and ribose-5-phosphate; these substrates are probably converted to glucose-6-phosphate before they are oxidized since leaf extracts contain an active fructose diphosphatase and a mechanism for the formation of hexose monophosphate from ribose-5-phosphate (244).

Reactions of Hexoses and Their Derivatives

The nonphosphorylated pathway of glucose oxidation.—No function for glucose dehydrogenase in animal tissues has yet been found. With a partially purified preparation from liver Nakamura (272) found galactose, D- and L-xylose, and D-arabinose to be oxidized, although more slowly than glucose, in confirmation of earlier studies of Strecker & Korkes (273). The preferential oxidation of the β-form of glucose by this enzyme has been confirmed by Nicolle *et al.* (274). Glucose dehydrogenase has recently been reported to occur in yeast on the basis of evidence obtained from a study of the inhibitory action of nicotine on glucose utilization (275). More direct confirmation for such an enzyme is required. Bruckmann (276) has reported the reduction of methylene blue by glucose in extracts of *Candida reukauffii*.

In microorganisms evidence continues to accumulate for the existence of a pathway of glucose oxidation involving nonphosphorylated derivatives: glucose→gluconic acid→2-ketogluconic acid→2,5-diketogluconic acid→citric acid cycle intermediates. Kulhánek (277) reported the formation of good yields of 2-keto acids from D-gluconate, L-idonate, and D-galactonate with strains of *Pseudomonas aeruginosa*. With *A. suboxydans* (278) a number of aldonic acids, including heptonic and pentonic acids, were oxidized and the generalization was drawn (279) that dehydrogenation in the 2-position requires the gluconate configuration in the first 3 carbon atoms, while the penultimate carbon atom may be oxidized when it has the D-configuration with a *cis*-hydroxyl in the adjacent position. According to Khesghi *et al.* (280), *A. suboxydans* produces only the 5-keto derivative from gluconic acid. Kulka & Walker (281) have demonstrated the formation of keto acids in 14 species of *Acetobacter*, 8 of which produced both 2- and 5-ketogluconate. Katznelson & Tanenbaum (282) have re-examined the pathway of maltose oxidation by *Acetobacter melanogenum* and concluded that hydrolysis to glucose precedes oxidation. No evidence for 5-ketogluconate as a precursor of 2,5-diketogluconate was obtained. With cell-free extracts of *Pseudomonas aeruginosa*, Claridge & Werkman (283) showed both glucose and gluconic

acid to be oxidized to 2-ketogluconate. This product was not utilized by the extracts, but radioactive 2-ketogluconate, incubated under aerobic conditions with cell suspensions of the organism, gave rise to a number of intermediates of the citric acid cycle. Malonic acid, glucose phosphate, and a number of unknown phosphate esters were also detected (284). While it is now rather conclusively established that the steps from glucose to 2-ketogluconate involve nonphosphorylated compounds, the nature of the subsequent steps is unknown. With extracts of *Aerobacter cloacae*, DeLey (285, 286) has obtained evidence for an adaptive 2-ketogluconokinase. The reaction product, which on hydrolysis yields 2-ketogluconic acid, is further metabolized by extracts of the same organism with the formation of 3-phosphoglyceric acid. However, organisms grown on glucose do not possess either 2-ketogluconokinase or the adpative kinase for gluconic acid, suggesting that the phosphorylation of these intermediates is not involved in glucose metabolism. The intermediates formed from glucose-6-phosphate have not been identified. Cells grown on galactonic acid yield extracts which will phosphorylate this substrate as well as gluconic acid and 2-ketogluconic acid (287). In *Pseudomonas fluorescens* the pattern of enzyme formation appears to differ from that found by DeLey. Narrod & Wood (288) have found kinases for both gluconate and 2-ketogluconate in glucose-grown cells. Preparations of these kinases have been obtained which have permitted the isolation of the phosphorylated products. The product of 2-ketogluconate esterification is further metabolized by extracts of the organism.

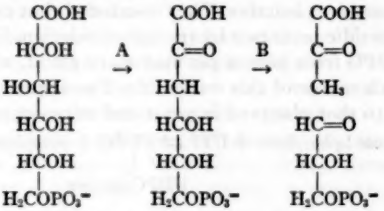
In the mold *Botrytis cinerea* gluconic acid formed by the oxidation of glucose appears to enter the oxidative pathway following esterification to phosphogluconate (289). Oxalic acid, which is accumulated by this organism, is most effectively formed from malic acid; oxalacetic acid and oxalsuccinic acid are poorer precursors. In *A. niger* the formation of gluconic acid from glucose is greatly enhanced by the presence of 0.02 *M* cyanide (290) which produces similar but smaller increases in the yield of citric and oxalic acids. These results might suggest the presence of a cyanide-resistant glucose oxidase in these organisms which resembles that of *Penicillium notatum*.

The question of gluconate metabolism in the homofermentative bacteria has been examined by Sokatch & Gunsalus (291). *Streptococcus faecalis* cells grown on glucose or on gluconate contain equivalent amounts of glucose-6-phosphate and 6-phosphogluconate dehydrogenases, but only gluconate-grown cells would ferment this substrate. The constitutive nature of 6-phosphogluconic dehydrogenase in this homofermentative organism is suggestive of a role for this enzyme in the synthesis of pentose phosphate.

The role of another oxidation product of glucose, glucosone, remains obscure. Becker (292) studied the metabolism of this compound in yeast and found it to inhibit glucose oxidation but not its fermentation. Glucosone itself was not fermented by intact cells but was metabolized by extracts fortified with ATP and DPN. The products have yet to be defined.

Other mechanisms of phosphogluconate metabolism.—The presence of glu-

cose-6-phosphate dehydrogenase is not sufficient evidence for the occurrence of the pentose phosphate pathway, since 6-phosphogluconate has been shown by Entner & Doudoroff (293) to undergo an alternate type of cleavage in which pyruvate and glyceraldehyde-3-phosphate are formed. The intermediate postulated by these workers has now been isolated and evidence has been obtained for the following reactions:



This sequence has now been established with enzymes from *P. saccharophila* (294) and *P. fluorescens* (295) and appears to occur as well in *Pseudomonas lindneri* (296). MacGee & Doudoroff (294) have developed procedures for the separation of the two steps in extracts of *P. saccharophila*. Phosphogluconic dehydrase (A) converts 6-phosphogluconic acid to the new phosphorylated derivative which was purified by barium fractionation and chromatography and isolated as the crystalline trisodium salt. It was identified as 2-keto-3-deoxy-6-phosphogluconic acid on the basis of a number of chemical and enzymatic properties. This product is decomposed by the splitting enzyme (B) to yield 1 mole each of pyruvic acid and glyceraldehyde-3-phosphate, which was identified by enzymatic methods. In an independent investigation, Kovachevich & Wood (295) isolated the same intermediate using extracts of *P. fluorescens*. Phosphogluconic dehydrase, separated from the splitting enzyme, was found to require both ferrous ions and glutathione for activity. The splitting enzyme was also purified and shown to cleave the intermediate under anaerobic conditions to form pyruvate and glyceraldehyde-3-phosphate. It is interesting to contemplate the potentialities of the new deoxy derivative, which would yield deoxyribose-5-phosphate on decarboxylation.

Resting cells of *P. lindneri* appear to ferment glucose by the classical Embden-Myerhof mechanism, since they produce 2 moles of ethanol and 2 moles of CO_2. However, in experiments with C^{14}-labeled glucose, Gibbs & DeMoss (296) showed that CO_2 is formed from C-1 and C-4 and the carbinol carbon atom of ethanol from C-2 and C-5. This pattern of glucose fermentation suggests a cleavage of the type found in *P. saccharophila* and *P. fluorescens*. Thus far, this type of cleavage has been reported only in *Pseudomonas* species, but it is to be expected that it will be found in other living forms.

The metabolism of galactose and mannose.—Interest in galactose metabolism in mammals continues to center about the synthesis of lactose and the function of the uridine nucleotide coenzymes. The synthesis of lactose from

C^{14}-labeled glucose was demonstrated in goat mammary gland *in vivo* by Reiss & Barry (297). Both fragments of lactose appear to be derived from blood glucose, and the galactose moiety is formed sufficiently rapidly to have the same specific activity as the glucose moiety. Kittinger & Reithel (298) were able to demonstrate lactose synthesis from glucose-1-phosphate and glycogen with a partially fractionated preparation from guinea pig mammary tissue. Glucose was inactive. They concluded that the galactose moiety arises from a glucosidic precursor by transglycosidation. Smith & Mills (299) have isolated UDPG from guinea pig mammary gland, which they found to be a relatively rich source of this nucleotide. The sequence of events is very probably similar to that observed in yeast and microorganisms (300).

$$\begin{array}{c}\text{glucose-1-phosphate} + \text{UTP} \rightleftarrows \text{UDPG} + \text{pyrophosphate} \\ \uparrow\downarrow \\ \text{UDPGalactose}\end{array} \qquad 12.$$

UDPG pyrophosphorylase (reaction 12) extracted from liver nuclei by Mills *et al.* (301) will also react with uridine diphosphate acetylglucosamine which is not cleaved by the yeast enzyme. Kalckar (302) has described a specific enzyme in *Hemophilus influenzae B*, which appears to hydrolyze UDPG to UMP[2] and glucose-1-phosphate. The function of this enzyme is not known.

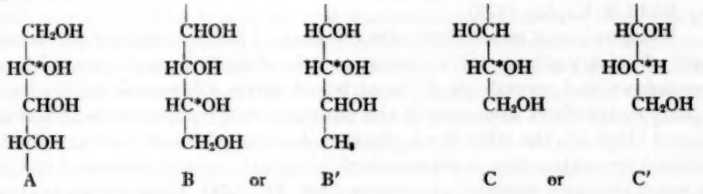
The participation of other coenzyme forms in lactose synthesis is suggested by the isolation of an unidentified derivative, neuraminelactose, from the mammary gland by Trucco & Caputto (303). Heyworth & Bacon (304) have also isolated a nitrogenous derivative of lactose which they believe may be identical with neuraminelactose. Galactose degradation in mammals does not appear to be an adaptive process (305).

More definitive information on the mechanism of the glucose-galactose transformation has been derived in studies with microorganisms. Hansen & Craine (306) studied the "waldenase" reaction in *Lactobacillus bulgaricus* extracts which had been freed of phosphoglucomutase and have estimated the equilibrium mixture to contain about 24 per cent of galactose-1-phosphate and 75 per cent of glucose-1-phosphate. Galactose metabolism appears in general to follow the pattern of glucose breakdown. However, in certain homofermentative organisms, Steele *et al.* (307) observed different proportions of fermentation products when the organism was grown on glucose or on galactose, including a shift from homolactic to heterolactic fermentation. These differences may be a reflection of differences in rate of fermentation. In yeast, during adaptation to galactose, aerobic fermentation appears only after a preliminary oxidation phase during which the R.Q. is unity; this phenomenon is interpreted in terms of an accumulation of intermediates and saturation of the respiratory systems (308).

A new nucleotide derivative has been isolated from bakers' yeast by Cabib & Leloir (309) and identified as guanosine diphosphate mannose. This compound, which is nonreducing, yields mannose on mild acid hydrolysis, and the residual nucleotide was shown to be guanosine-5′-diphosphate. It was postulated that this compound was involved in the synthesis of mannan

present in the yeast cell wall. This compound, as well as a number of other nucleotide derivatives containing additional carbohydrate, have been isolated by Strominger (310) from hen oviduct extracts. With respect to the biological origin of the carbon chain of mannose, the results of Sowden *et al.* (266) with *T. utilis* suggest a mechanism involving exclusion of carbon atom 1, although earlier experiments of Gilvarg (311) indicate the direct conversion of glucose to yeast mannose. The formation of mannose from smaller fragments is also suggested by the finding of Eagen & Randles (312) that *P. fluorescens* forms a mannose polysaccharide only when the organism is grown on a number of intermediates of the tricarboxylic acid cycle or of glycolysis, but not when grown on hexoses or pentoses.

The oxidation of polyols.—Edson (313) has recently reviewed the work in which he and his collaborators have characterized three polyol dehydrogenases of different specificities. These appear to require the following configuration:



The rat liver enzyme of Blakley (314) was re-examined (315) and found to oxidize pentitols, hexitols, and heptitols which conform to configuration A, where the carbinol indicated with the asterisk is converted to the keto group. The configuration of the hydroxyl group on carbon atom 3 may be either D or L; the others must have the configuration indicated. The specific DPN requirement and the reversibility of the reaction were confirmed. The name L-iditol dehydrogenase was suggested since L-iditol contains the specific configuration at both ends of the molecule. A similar enzyme has been purified about 200-fold from sheep liver (316). Independently, Williams-Ashman & Banks (317) have reported the purification of this enzyme from rat liver (70-fold) and seminal vesicle and coagulating gland (10-fold). On the basis of the distribution of this enzyme in accessory sexual tissues, they suggest that it may function in the formation of seminal fructose.

Another polyol dehydrogenase present in *A. suboxydans* oxidizes substrates having the B or B′ configuration; the site of oxidation is marked. Extracts of this organism contain a DPN-linked dehydrogenase which oxidizes mannitol and sorbitol to fructose (318, 319). A second enzyme, with lower pH optimum, showed a substrate specificity corresponding to the Bertrand-Hudson rule. DPN was not required. This enzyme was named D-mannitol dehydrogenase.

Substrates of type C are oxidized by extracts of *Pseudomonas sp.* grown on appropriate substrates (320). This enzyme also required DPN and was

named galactitol dehydrogenase. Hexoses and pentoses bearing the *trans*-configuration (either D or L) were oxidized.

While *A. suboxydans* will utilize polyols for growth, these cannot be replaced by their oxidation products, such as dihydroxyacetone or sorbose (321). King & Cheldelin have found that ketoses are oxidized by resting cells and that dinitrophenol, which is without effect on growth on glycerol or sorbitol, will inhibit this oxidation. The polyol oxidation mechanism appears to function primarily as an energy source.

Wick *et al.* (322) found that radioactive mannitol forms CO_2 when given orally or injected into the portal vein of mammals. This may be attributable to the action of liver L-iditol dehydrogenase, which slowly attacks this substrate (316). These enzymes may also account for adaptive enzyme patterns in *Lactobacillus casei* (323, 324).

The formation of hexitol phosphate from fructose-6-phosphate with a DPN-linked enzyme prepared from extracts of *E. coli* has been demonstrated by Wolff & Kaplan (325).

Glucuronic acid metabolism.—On the basis of earlier work the glucuronic acid of urinary glucuronides appears to arise directly from hexose, without breakdown and resynthesis of the carbon skeleton. Glucuronic acid is itself poorly metabolized, appearing in the urine as a conjugate or as the free compound (326). On the other hand, glucuronolactone (glucurone) is very poorly utilized for conjugation or polysaccharide formation and is converted to CO_2 in much the same manner as is glucose (326, 327, 328). These results suggest that the lactone may be readily reducible to glucose. Evidence for the formation of L-xylulose from glucuronolactone has been obtained by Touster *et al.* (329, 330). This sugar was detected in animals after glucuronolactone feeding as well as in a normal human subject. L-Xylulose is rapidly utilized by liver slices; however, its metabolic role and the mechanism of its formation from glucuronolactone are not known.

Considerable progress has been made in elucidating the mechanism of glucuronic acid formation from glucose. Shirai & Ohkubo (331) have demonstrated the synthesis of glucuronides by rat liver slices and shown this to be augmented by the addition of glucosides, such as glycogen or glucose-1-phosphate. Dutton & Storey (332) have demonstrated the existence of a factor which is essential for glucuronide synthesis in mouse liver homogenates and obtained evidence for the nucleotide nature of this factor (333). Strominger *et al.* (334) have isolated a DPN-linked enzyme from rat liver which catalyzes the oxidation of UDPG to a glucuronide nucleotide derivative. This nucleotide is considered to be identical with the liver factor of Dutton & Storey.

Glucosamine.—The first direct demonstration of glucosamine synthesis was that of Leloir & Cardini (335) with an extract of *Neurospora* mycelium. Either glucose-6-phosphate or fructose-6-phosphate served as substrate and glutamine as the donor. The product, which was capable of acetylation by a *Neurospora* enzyme, was provisionally identified as glucosamine-6-phosphate. The same product was obtained starting with glucosamine, which was

esterified by the extracts. Acetylglucosamine, on the other hand, could not be converted to the phosphate ester. While the hexokinases of brain and yeast will phosphorylate glucosamine, Bueding *et al.* (336) have found that a specific glucosamine kinase present in the trematode *Schistosoma mansomi* will react with this substrate and relate the toxicity of glucosamine for the organism to the activity of this enzyme. Bollet *et al.* (337) have observed the synthesis of glucosamine in minced connective tissue incubated in the presence of a serum ultra-filtrate and glucose. In studies on the degradation of glucosamine by *E. coli*, Oka & Murachi (338) have obtained evidence that deamination to glucose precedes oxidation or fermentation.

Ascorbic acid.—The effect of chloretone (1,1,1-trichloro-2-methyl-2-propanol) in stimulating the urinary excretion of ascorbic acid in the rat has been examined by Burns *et al.* (339). On the basis of the rate of turnover of C^{14}-labeled ascorbic acid they concluded that chloretone and pentobarbital increased the rate of ascorbic acid biosynthesis, although, in untreated animals only 15 per cent of ascorbic acid was excreted in unaltered form. Horowitz & King (340) injected uniformly labeled glucuronolactone into chloretone-treated rats and found essentially uniform labeling in the ascorbic acid excreted. Since the product was 4 to 8 times more active than that obtained from glucose-C^{14}, the authors conclude that glucurone is closer than glucose to ascorbic acid. However, the large dilution suffered by injected glucose on equilibration with the glucose pool renders this conclusion uncertain. Some interesting observations on the effect of chloretone have been made by Ganguli *et al.* (341). They found that in animals deficient with respect to any one of several of the B-vitamins the effect of chloretone on ascorbic acid excretion is entirely suppressed. Chloretonized animals given labeled pyruvamide excreted labeled ascorbic acid, although glycogen contained little activity and none could be detected in glycolysis or Krebs cycle intermediates. These results are in contradiction to earlier evidence for the direct conversion of glucose.

Isherwood *et al.* have proposed a dual pathway for L-ascorbic acid synthesis as shown in Figure 3. Support for this scheme has been obtained by testing a large number of possible precursors (342). Increases in L-ascorbic acid content of cress seedlings followed feeding of II, III. V, and VI, while D-altrono-γ-lactone gave rise to D-araboascorbic acid, and other closely related derivatives including L-idono-γ-lactone and L-talono-γ-lactone were inactive. Similar responses to subcutaneous injection were obtained in the rat, except that D-altrono-γ-lactone was inactive, while D-araboascorbic acid was produced from D-mannono-γ-lactone. L-Sorbose, D-sorbitol, and D-gluconic acid were not utilized for ascorbic acid synthesis under these conditions. D-Glyceric acid, which was formed together with ascorbic acid in cress seedlings, did not satisfy the requirements of a precursor or an intermediate (343). An important advance was made by Mapson *et al.* (344) with the demonstration that a particulate enzyme preparation from pea seedlings would convert L-galactono-γ-lactone (VI) to L-ascorbic acid. The activity was associated

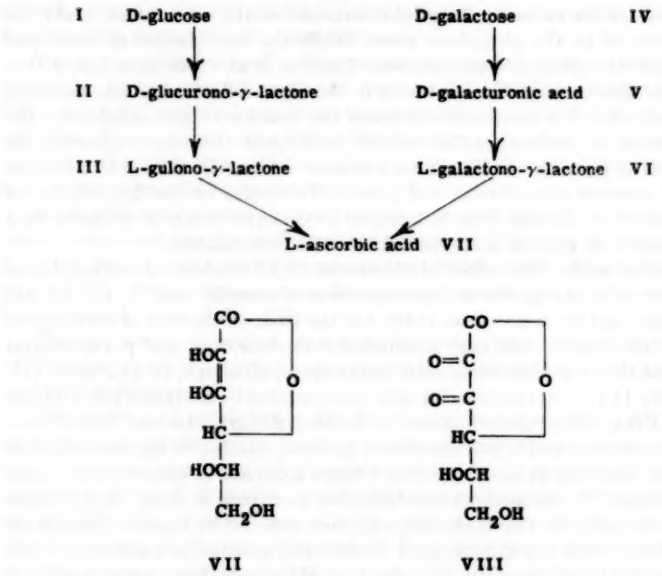


FIG. 3. Proposed pathway for L-ascorbic acid synthesis.

with the mitochondrial fraction. Inhibition studies indicated that the cytochrome system participated as the terminal oxidase in this system. Phosphorylation reactions are apparently not involved since ATP was not needed and dinitrophenol failed to inhibit the conversion. D-Gulono-γ-lactone (III), which was active in intact cress seedlings, did not give rise to L-ascorbic acid with the pea mitochondrial preparation. However, D-altrono-γ-lactone was converted to D-araboascorbic acid. Similar experiments with III and IV with extracts from animal tissues are reported to be in progress. The participation of 2-ketogulonic acid as an intermediate in ascorbic acid synthesis is indicated by the work of Yashima (345), who studied this conversion in muscle extracts.

Ascorbic acid is known to exist as the ene-diol (VII), which on oxidation yields dehydroascorbic acid (VIII). The latter is formed as an end product of ascorbic acid oxidase action. However, evidence has recently been obtained as a result of work in three laboratories (346, 347, 348) which supports the view (349) that an intermediate form, monohydroascorbic acid, exists. An intermediate of this or similar nature participates in the oxidation of DPNH by a cyanide sensitive system from plants which is activated by a combination of ascorbic acid and ascorbic acid oxidase. Dehydroascorbic acid is inactive in this system.

The Metabolism of Pentoses and Heptuloses

The formation of hexose phosphate from pentose phosphate.—The metabolism of pentose phosphate in animal and plant tissues, as well as in certain microorganisms, now appears to involve a prior conversion to hexose monophosphate. These reactions have been reviewed in a previous section and are summarized in Figure 2. With extracts of rat liver (237) and pea root (244) hexose monophosphate formed from 1-C^{14}-ribose-5-phosphate contains isotope in carbon atoms 1 and 3 in roughly the proportions predicted by the reactions indicated. Comparable results were obtained with 2,3-labeled pentose phosphate. Pea leaf extracts yielded similar results except that in this case 1-labeled pentose phosphate gave appreciable labeling in carbon atoms 4 and 6, indicating the presence of an additional mechanism absent in liver or pea root. The appearance of isotope in four carbon atoms of the hexose when only carbon atom 1 of the pentose was labeled is of interest in view of the rapid formation of essentially uniformly labeled hexose during photosynthesis in $C^{14}O_2$.

With liver slices, Katz *et al.* (350) obtained quantitatively different results with ribose-1-C^{14}. Glucose isolated from the slices was found to be equally labeled in carbon atoms 1 and 3, suggesting its direct formation by the transketolase-transaldolase sequence (reaction 7) without secondary hexose formation from tetrose phosphate (reaction 11).

Pentose fermentation in microorganisms.—In several instances the results of bacterial fermentations can be explained in terms of the formation of hexose which is in turn metabolized by the Embden-Meyerhof pathway. Neish & Simpson (351) found that under anaerobic conditions *A. aerogenes* fermented arabinose-1-C^{14} with the formation of lactate which was labeled in both methyl and carboxyl carbons, with twice as much activity in the former. Formate and CO_2 were also highly labeled. These results may now be interpreted as indicating that in this organism 1,3-labeled glucose arises from 1-labeled pentose and is then fermented to yield 1,3-labeled lactate. Gibbs (352) has obtained similar results with yeast extracts, which account for the earlier observations of Dickens (353) on the fermentation of pentose phosphate. With 1-labeled ribose phosphate isotope appears in the CO_2 formed, as well as in the methyl carbon atom of the ethanol, with the latter approximately three times more active. This result, again, is consistent with the formation of 1,3-labeled hexose phosphate as an intermediate in pentose phosphate fermentation. A similar conclusion is to be drawn from the data which Gibbs *et al.* (354) have obtained for the anaerobic dissimilation of D-xylose and D-arabinose in *E. coli*, where, with 1-C^{14}-labeled pentose, isotope appears not only in the methyl groups of acetic acid and lactic acid, but also in the carboxyl group of lactic acid and in formic acid. Bergmann *et al.* (355) have shown the metabolism of pentoses by *E. coli* to be an adaptive process and have demonstrated the formation of phosphoglyceric acid under aerobic and anaerobic conditions. With cell-free extracts of these organisms they found (247) a rapid utilization of ribose-5-phosphate whether the cells were

grown on ribose, xylose, arabinose, or glucose. D-Arabinose-5-phosphate was not attacked, although xylose-5-phosphate and xylose-1-phosphate were slowly utilized. These esters have not previously been shown to have biological activity.

In other organisms pentose fermentation does not involve the intermediate formation of hexose. The mold *Fusarium lini* appears to follow a pathway in which pentose is cleaved to C_2 and C_3 products. Gibbs *et al.* (356) have obtained conditions in which nearly equivalent quantities of CO_2, ethanol, and acetic acid are produced. With 1-C^{14}-xylose only the acetic acid methyl group is labeled.

In the case of arabinose and xylose isomerization to the corresponding keto sugars precedes formation of the phosphate esters. Hochster & Watson (357) have demonstrated the reaction:

$$\text{D-xylose} \rightleftarrows \text{D-xylulose} \qquad 13.$$

with a preparation from xylose adapted cells of *Pseudomonas hydrophilat.* The equilibrium mixture contained 16 per cent of xylulose, but the reaction could be shifted to the right by the addition of borate. Lampen (358) has extended his previous studies with *Lactobacillus pentosus* to L-arabinose and demonstrated that cells grown on this sugar contain an isomerase which forms L-ribulose. This organism forms acetate from C-1 and C-2 of pentose and lactate from C-3, C-4, and C-5. Bernstein (359) has confirmed earlier observations of Lampen *et al.* (360) indicating that C-1 gives rise to the methyl group of acetate and C-3 forms the lactate carboxyl group.

The mechanism of pentose fermentation in the propionic acid bacteria remains obscure. Wood & Leaver (361) have found the products of pentose fermentation by these organisms to consist of proportions of CO_2, succinate, propionate, and acetate similar to those obtained with hexose. Rappaport & Barker (362) employed 1-C^{14}-arabinose and found all the carbon atoms of the fermentation products to contain significant quantities of C^{14}. On the basis of this evidence and previous results it is postulated that the fermentation involves succinic acid as an intermediate; however, the initial steps in carbohydrate breakdown in these organisms cannot yet be evaluated.

The formation of C_2 compounds.—In some of the bacterial fermentations discussed in the preceding section there is a conversion of carbon atoms 1 and 2 of pentose to acetate. This may involve the formation of "active acetate" (i.e., acetyl CoA) from "active glycolaldehyde," although no evidence for such a transformation is available. Sprinson & Weliky (363) have suggested such a reaction to account for their observation that D-ribose or D-xylose in the intact rat is an effective acetyl precursor, with C-1 of the pentose contributing the methyl group of the acetate. However, this result can be interpreted equally well in terms of prior glucose formation from pentose by the transketolase and transaldolase reactions. In similar experiments Greenberg & Sassenrath (364) failed to observe the formation of glycine from 1-C^{14}-labeled pentose. The incorporation of glycolaldehyde into pentose

by way of xylulose-1-phosphate and ribulose phosphate has been suggested by McGeown & Malpress (365), but further experimental evidence for this conclusion is necessary.

Heptulose occurrence and metabolism.—With uniformly labeled sedoheptulose, isolated from *Sedum spectabile* kept in an atmosphere of $C^{14}O_2$, Tolbert & Zill (366, 367) followed the metabolism of this substance in intact sugar beet leaves. In the light and the presence of air sucrose was the major product, while in the dark glutamic acid, aspartic acid and succinic acid were formed. In the light under nitrogen chromatographic evidence for a tetrose intermediate was obtained.

Mannoheptulose, long known to occur in the fruit of the avocado, has been found by Nordal & Benson (368) to occur as a phosphate ester in the leaves of this plant. This sugar may arise in the transaldolase reaction under conditions which would favor formation of the *cis*-linkage, rather than the *trans*-linkage present in positions 3 and 4 of sedoheptulose.

The first demonstration of heptulose in intact mammalian tissue has been made by Peeters *et al.* (369), who detected a compound with the same R_F as sedoheptulose in colostrum and in lactating udder tissue.

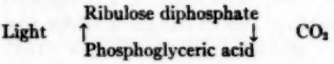
Pentose precursors of nucleic acid.—Klenow (161) has isolated ribose-1,5-diphosphate as an intermediate in the phosphoribomutase reaction and obtained evidence, in collaboration with Emberland & Plesner (370), for the formation of adenosine-5′-phosphate from this substance. Saffran & Scarano (371, 372) have obtained similar results employing ribose-5-phosphate and ATP to generate ribose-1,5-diphosphate. However, Kornberg *et al.* have demonstrated that a new ester, 5-phosphoribosylpyrophosphate (373), which arises from ATP and ribose-5-phosphate, is an intermediate in 5-nucleotide synthesis (373, 374). The possibility remains that ribose-1,5-diphosphate can be converted to 5-phosphoribosylpyrophosphate.

THE PATH OF CARBON IN PHOTOSYNTHESIS

Earlier work on the intermediates in carbohydrate synthesis in photosynthesis has been reviewed in previous volumes of this series and in the Annual Review of Plant Physiology. A number of important facts have been established which provided the basis for recent studies. These may be summarized as follows: (*a*) Photosynthesis consists of two separable phases including (*i*) the photolysis of water and the production of chemical energy and (*ii*) the fixation of carbon dioxide. It was evident that with the proper acceptor the latter process would become independent of light. (*b*) Experiments with $C^{14}O_2$, carried out mainly at the University of California by Calvin and his collaborators and at the University of Chicago by Gaffron, Fager, and Rosenberg had established that phosphoglyceric acid is the earliest detectable product of photosynthesis. The carboxyl group becomes labeled very rapidly, but isotope soon appears in the α- and β-carbon atoms which are always equally radioactive. A similar pattern was found in the hexose esters, which become labeled first in positions 3 and 4, later in the

other carbon atoms, which again are equally labeled. (*c*) The phosphate esters of ribulose and sedoheptulose become labeled very early and on the basis of kinetic studies appear to participate in the synthesis of phosphoglyceric acid.

The carboxylation reaction.—The relationship of these intermediates in steady state photosynthesis in *Scenedesmus* was studied by Calvin & Massini (375) who made the significant observation that, when the light was turned off, the concentration of phosphoglycerate increased, while that of the sugar diphosphates (mainly ribulose diphosphate) decreased. Later, Bassham *et al.* (376) found the opposite result to be produced when the flow of carbon dioxide was interrupted but illumination continued. It was suggested that the phosphate esters of ribulose and sedoheptulose could provide the C_2 acceptor for CO_2 and were thus precursors of phosphoglycerate. Light would provide energy needed to regenerate the pentose and heptulose esters. A scheme was proposed (377) in which ribulose diphosphate acted as the immediate precursor of phosphoglyceric acid.



Thus, removal of CO_2 would increase the quantity of ribulose diphosphate at the expense of phosphoglyceric acid, while in the dark phosphoglyceric acid would accumulate and the ribulose diphosphate level would fall.

Direct evidence for the role of pentose phosphate as a precursor of phosphoglyceric acid is now provided by the independent observations of Quayle *et al.* (377), Weissbach *et al.* (378), and Horecker (379). The former group employed as substrate an eluate from the ribulose diphosphate area of paper chromatograms and found that when this material was added to cell-free extracts of *Chlorella* in the dark, CO_2 was fixed in the carboxyl group of phosphoglyceric acid. Weissbach *et al.* (378) employed a soluble extract of spinach leaves with ribose-5-phosphate as the substrate and also observed CO_2 fixation in the carboxyl group of phosphoglyceric acid. Other substrates were inactive, except glucose-6-phosphate which showed slight activity. With 1-C^{14}-ribose-5-phosphate most of the isotope in the phosphoglyceric acid was in the β-carbon atom. Carbon dioxide fixation was stimulated by TPN and ATP. From these spinach extracts a kinase was purified which catalyzed a reaction between ATP and ribose-5-phosphate. The product has been isolated and identified as ribulose diphosphate (380). This ester is more effective than ribose-5-phosphate as a substrate for the carboxylation reaction with spinach extracts and ATP or TPN are no longer required (381). Calvin (382) has proposed a mechanism involving the direct carboxylation of ribulose diphosphate to form a branched six carbon compound which is tautomeric with a phosphoglycero-phosphoglycerate condensation product. On hydrolysis this would yield 2 moles of phosphoglyceric acid. The disappearance of the TPN requirement, when ribulose diphosphate replaces ri-

bose-5-phosphate as the substrate, renders unlikely a mechanism in which glyceraldehyde-3-phosphate is an intermediate.

The regeneration of the CO_2 acceptor.—Bassham *et al.* (376) have developed methods which have permitted them to determine the distribution of C^{14} in the C_6, C_5, and C_7 compounds following very brief exposure to $C^{14}O_2$ and on the basis of these results have proposed a cycle for carbon reduction in photosynthesis. A modified version of this scheme, based on known enzymatic mechanisms, is given in Figure 4.

The isotope distribution which would result from this series of reactions is precisely that found by Calvin & Massini (375) and Bassham *et al.* (376). Hexose phosphate would be labeled first in carbon atoms 3 and 4, and after each cycle this isotope would appear in the other carbon atoms of hexose as well as in the α- and β-carbon atoms of phosphoglyceric acid. Sedoheptulose phosphate would first become labeled in carbon atoms 3, 4, and 5, and these would be essentially equal in isotope content. Ribulose phosphate would soon become labeled in the first three carbon atoms with heaviest labeling in carbon 3, since all three pentose phosphate molecules formed contain isotope in this position, whereas only one is labeled in carbon atoms 1 and 2. Furthermore, carbon atom 3 of pentose contains the aldehyde group of glyceraldehyde-3-phosphate, which arises from the carboxyl group of phosphoglycerate and would be expected to be labeled earlier than carbon atoms 3 and 4 of the hexose monophosphate. Bassham *et al.* found carbon atom 3 of the ribulose phosphate to contain six times as much radioactivity as carbon atom 1 and 2 after eight seconds photosynthesis in $C^{14}O_2$.

The cycle also accounts for the fact that the α- and β-carbon atoms of phosphoglyceric acid always contain equal amounts of isotope, since the labeled carbon atoms both arise from C-3 of hexose.

In the presence of carbon dioxide, ribulose diphosphate is converted to phosphoglyceric acid. The conversion of this product to glyceraldehyde-3-phosphate by triose phosphate dehydrogenase requires both ATP and reducing power, and ATP is also required for the formation of ribulose diphosphate from the monophosphate. Vishniac & Ochoa (383) have demonstrated reduction of pyridine nucleotides associated with the photolysis of water, as well as a coupled esterification of phosphate in the reoxidation of the photochemically reduced coenzyme. Arnon *et al.* (384), using intact spinach chloroplasts, and Frenkel (385), using particles from *Rhodospirillum rubrum*, have observed photochemical phosphate esterification. While it is not certain that this represents anything more than phosphorylation accompanying a photochemically stimulated oxidation such as that observed by Vernon & Kamen (386), it is apparent that, whether directly or indirectly, light will lead to the synthesis of ATP. Takashima & Mitsui have observed a photochemical transfer of phosphate (387) from glycerophosphate to a labile ester in spinach homogenates which may represent an alternate mechanism for regenerating phosphate donors. These effects of light and CO_2 may account for the ribulose diphosphate-phosphoglyceric acid relations discussed previously.

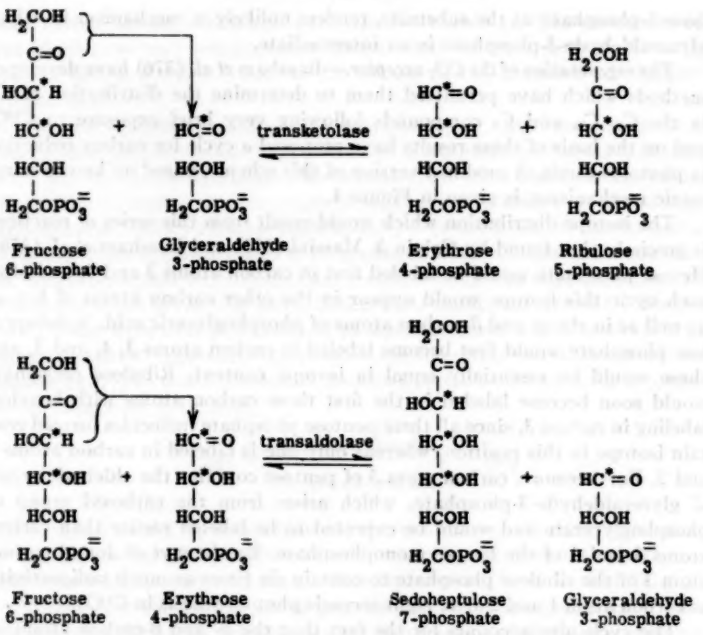


FIG. 4. Proposed cycle for carbon reduction in photosynthesis.

Although the carboxylation system is readily obtained in soluble form, Fager (388) has obtained evidence that it is associated with the intact chloroplast. Spinach chloroplasts showed CO_2 fixation in phosphoglyceric acid which was stimulated by the addition of a boiled algal extract and was increased by illumination. This system is sensitive to sulfhydryl reagents, a property which is shared by the isolated phosphopentokinase (380).

An alternate route to phosphoglyceric acid has been suggested by Stafford *et al.* (389) who found a specific enzyme in plant extracts which catalyzes the reduction of hydroxypyruvate to D-glyceric acid with DPNH. However, they point out the need for further evidence for supporting reactions before this active, widely distributed enzyme can be assigned a role in plant metabolism.

In view of the great bulk of evidence for phosphoglyceric acid as the primary product of CO_2 fixation in photosynthesis, it is difficult to interpret the report of Doman (390) who claims to have obtained evidence for two unidentified substances, neither of which is phosphoglyceric acid, as primary product of photosynthesis.

Other carboxylation mechanisms.—There is little reason to believe that

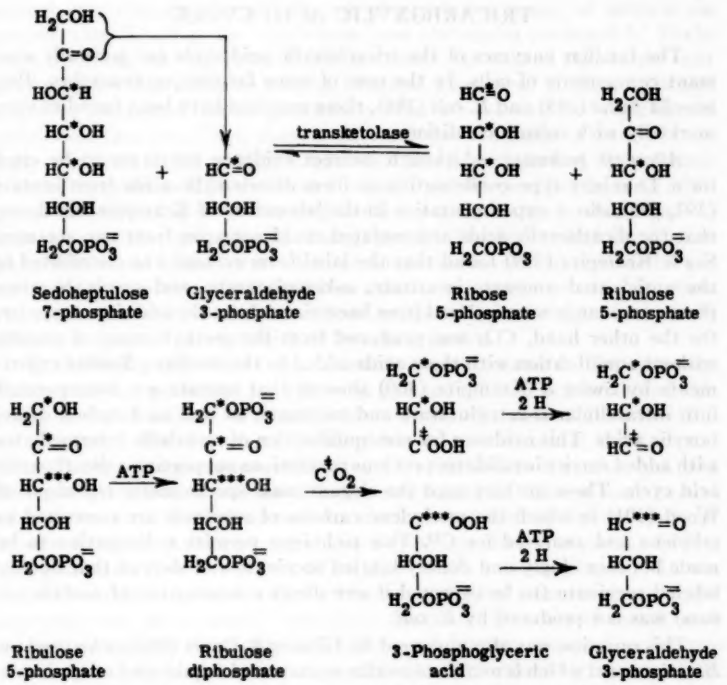


FIG. 4 (continued)

other carboxylation reactions participate in the primary mechanism of carbon dioxide reduction in photosynthesis. Malic acid becomes labeled early but does not fulfill the requirements of a carbohydrate precursor (375, 376, 391). Other components of the citric acid cycle do not appear until the light is turned off (375). Bean & Hassid (392) found the products of the early stages of photosynthesis in a red alga to be identical with those produced in green plants, although glycerol phosphate and α-D-galactopyranosylglycerol also accumulated. Siegel (393) obtained evidence for a light-induced carboxylation of acetone to acetoacetate in resting cells of *Rhodopseudomonas gelatinosa*, which he proposes to represent a second function for radiant energy in this organism. Krotkov *et al.* (394) studied the synthesis of glucose and starch in tobacco and found a number of labeled precursors, such as formate, acetate, and lactate to be incorporated only in the light. The distribution of isotope in the hexose was that expected from patterns established in heterotrophic organisms, and the function of light in this system is not clear.

TRICARBOXYLIC ACID CYCLE

The familiar enzymes of the tricarboxylic acid cycle are generally constant components of cells. In the case of some facultative anaerobes, *Pasteurella pestis* (395) and *E. coli* (396), these enzymes have been found to vary markedly with culture conditions.

Alternate pathways.—Although indirect evidence continues to be cited for a Thunberg-type condensation to form dicarboxylic acids from acetate (397, 398), direct experimentation in the laboratory of Krampitz has shown that the dicarboxylic acids accumulated could not arise from two acetates. Saz & Krampitz (399) found that the label from acetate was distributed in the anticipated amounts in citrate, α-ketoglutarate, and succinate when these compounds were isolated from bacteria without the addition of carrier. On the other hand, CO_2 was produced from the methyl group of acetate without equilibration with these acids added to the medium. Similar experiments by Swim & Krampitz (400) showed that acetate was incorporated into intracellular α-ketoglutarate and succinate, as well as 4-carbon dicarboxylic acids. This evidence for nonequilibration of metabolic intermediates with added carrier invalidates previous conclusions supporting a dicarboxylic acid cycle. These authors used the elegant mass spectrometer technique of Wood (401) in which the methylene carbons of succinate are converted to ethylene and analyzed for C^{13}. This technique permits a distinction to be made between singly and doubly labeled succinate and showed that doubly labeled succinate (to be expected if any direct condensation of acetate occurs) was not produced by *E. coli*.

This question was also answered by Gilvarg & Davis (402), who used an *E. coli* mutant which is unable to oxidize acetate and requires α-ketoglutarate or glutamate for growth. All of the enzymes of the tricarboxylic acid cycle are present in extracts of these cells with the exception of the citrate condensing enzyme. It was also shown that this strain does not produce CO_2 from the methylene carbons of succinate. It was concluded that acetate is oxidized only by the tricarboxylic acid cycle in this organism. Wheat & Ajl (403) also found the component enzymes of the citric acid cycle in *E. coli*.

Isotope studies with *E. coli* under growth conditions with glucose or acetate as substrate were interpreted by McQuillen & Roberts (404, 405) as showing that the tricarboxylic acid cycle in this organism is used primarily for synthesis of assimilation products.

α-Ketoglutarate formation.—Some recent isotope experiments have shown that reactions outside the tricarboxylic acid cycle can alter distribution patterns and that still other pathways must be considered. Hendler & Anfinsen (406) measured incorporation of $C^{14}O_2$ into glutamic acid by preparations of hens' oviduct. In addition to the anticipated fixation in the α-carboxyl group, as much as 30 per cent of the total radioactivity of glutamic acid was found in the γ-carboxyl. Control experiments showed that succinate carboxyl label did not appear in this position of glutamate. An explanation was

offered involving fixation of CO_2 into the carboxyl groups of acetate via acetoacetate. Much greater deviations from the results predicted by Krebs cycle reactions were found by Tomlinson (407). He showed that 90 per cent of $C^{14}O_2$ fixed into glutamic acid by *C. kluyveri* appeared in the γ-carboxyl. Opposing the mechanism suggested for oviduct are the results of experiments with labeled acetate, which showed acetate carboxyl appearing mainly in the α-carboxyl and β-carbon of glutamic acid. The fact that very little acetate carboxyl appears as CO_2 when metabolized by *C. kluyveri* is offered as additional evidence that the citric acid cycle is not operating in this organism even for synthetic purposes. It is suggested by Tomlinson that the label in glutamate could come from α-ketoglutarate via isocitrate produced by a reverse aconitase, one which dehydrates citric acid on the opposite side from the conventional aconitase. There is no evidence, however, that any of the tricarboxylic acids are metabolized by this organism.

Evidence for the degradation of succinate to acetate by an unknown mechanism was presented by Topper & Stetten (408). They incubated carboxyl-labeled succinate with rabbit liver slices, with sulfanilamide to trap acetyl groups. The acetyl groups were labeled exclusively in the carboxyl group. Control experiments eliminated CO_2 fixation as a mechanism, and this conclusion was strengthened by an experiment in which a large part of deuterium on the methylene carbons was found to accompany the carboxyl groups. Reversal of the tricarboxylic acid cycle was eliminated by the finding that glutamate had 98 per cent of its label in the α-carboxyl group. It was noted that relatively more acetyl groups were derived from succinate under anaerobic than under aerobic conditions. Since all of the known reactions of succinate appeared to be eliminated, Topper & Stetten proposed a cleavage of a dicarboxylic acid as the means of producing carboxyl-labeled acetate. This type of explanation does not eliminate the possibility of prior condensation of succinic acid with another molecule to produce the actual precursor.

Enzymes of the Tricarboxylic Acid Cycle

Citrate formation.—The formation of citric acid in a variety of preparations has been shown to proceed via the condensing enzyme, or at least to incorporate isotope in a pattern compatable with the condensation of oxalacetate with acetyl CoA. With *A. niger* the condensing enzyme has been demonstrated and purified 50-fold (409). Isotope experiments support this mechanism (398, 410) with the complication in the steady state of extensive recycling. Condensation of acetyl CoA has also been implicated in spinach (411), prostate (412), mammary gland (413), *P. pestis* (414), *P. chrysogenum* (415 to 418), *Pseudomonas* (419, 420), the mold *Blastiocladiella emersonii* (421), and the avian tubercle bacillus (422). Coxon (423) has concluded that only a single pathway for pyruvate oxidation, via citrate, occurs in pigeon brain preparations. Enzymes of the tricarboxylic acid cycle, including a low level of condensing enzyme, were found in extracts of lupine leaves (424). The purified condensing enzyme of *A. niger* was found to be inhibited by

Mg^{++} (425). It is possible that this inhibition is only apparent and caused by stimulation of a citridesmolase.

It has been well established that citric acid cycle enzymes occur in mitochondria. Sisakian & Mosolova (426) reported that they also occur in chloroplasts, and Dallam & Taylor (427) found certain of these enzymes associated with a lipoprotein fraction from cytoplasmic granules.

The acetate activating enzyme of heart muscle was purified by Hele (428), who found the reaction

$$\text{ATP} + \text{CoA} + \text{acetate} \rightleftarrows \text{AMP} + \text{acetyl CoA} + \text{pyrophosphate} \qquad 14.$$

to have an equilibrium constant near unity at neutral pH.

Degradation of tricarboxylic acid.—The formation of acetate and oxalacetate from citrate by *S. faecalis* was shown in 1953 by Gillespie & Gunsalus (429). Similar studies by Dagley (430) and by Wheat, Wong & Ajl (431) showed this cleavage to be catalyzed by enzymes from *A. aerogenes* and *E. coli*. The enzyme from *coli*, as was shown for *S. faecalis*, does not require CoA or any other factor adsorbed by Dowex 1, but does require a cation (Mg^{++}) as does the *S. faecalis* enzyme (Mn^{++}). The formation of acetate from citrate does not result from a reversal of the citrate condensing enzyme followed by hydrolysis, since CoA is not involved. While it is probable that a completely separate enzyme is involved, citridesmolase free from condensing enzyme has not been reported.

The anaerobic conversion of citrate to succinate, acetate, and CO_2 by preparations from *Bacterium succinum* was described by Takahashi (432). This system is inhibited by α, α'-dipyridyl and fixes $C^{14}O_2$ into the carboxyl groups of succinate. DPN and Mg^{++} are required, and CoA and ATP stimulate. The system is adaptive and does not parallel condensing enzyme activity. It was suggested that citrate is split to oxalacetate and acetate by an enzyme different from the condensing enzyme, but that ATP and CoA might play a role in activating citrate. A scheme for the formation of succinate simultaneously by an oxidative and reductive pathway also proposed by Takahashi (433) offers another locus for these cofactors to be used, the oxidation of α-ketoglutarate. In this scheme the two oxidative steps, isocitrate and α-ketoglutarate oxidations, are coupled with oxalacetate and fumarate reduction, so that two citrates yield two succinates, two CO_2, and one acetate.

In 1953 Campbell, Smith & Eagles (434) described the formation of glyoxylate and succinate from citrate by *Pseudomonas aeruginosa*. The mechanism of this reaction was investigated simultaneously in three laboratories. Both Smith & Gunsalus (435) and Saz (436) used extracts of *P. aeruginosa* and demonstrated that isocitrate is the actual substrate for the splitting reaction. Smith & Gunsalus were unable to demonstrate reversal of this reaction, but both Saz (436) and Olson (437), using extracts of *P. chrysogenum*, claim that the reaction is reversible. Smith & Gunsalus find Mg^{++} necessary, and, in addition, Saz found a stimulation by a dialyzable organic factor in yeast extract. The cleavage enzyme has been named "isocitritase" and "d-iso-

citric lyase." In addition to the organisms listed above, baker's yeast, *A. niger* and *Rhizopus* contain this activity (437).

The oxidation of tricarboxylic acids has long been known to proceed via isocitric dehydrogenase with TPN as oxidant. Several years ago Kornberg & Pricer (438) described an enzyme from yeast which requires DPN instead of TPN. Similar enzymes have been found by Plaut & Sung (439) in animal tissues and partially separated from the TPN-requiring enzyme. The purified DPN-isocitric dehydrogenase resembles the yeast enzyme in requiring Mg^{++} or Mn^{++} for activity but differs in lacking a requirement for adenylic acid. The product of the oxidation was identified as α-ketoglutarate. The formation of α-ketoglutarate and CO_2 by the TPN enzyme is freely reversible, and since DPN and TPN have essentially identical oxidation-reduction potentials, the same equilibrium must be attained. Nevertheless, with the animal as well as with the yeast DPN-requiring enzyme no reversal of isocitrate oxidation could be detected. Therefore it is necessary to postulate a peculiar inhibition of the reverse reaction, as was found in the transhydrogenase reaction, or the participation of additional components in the over-all reaction. A suggestion favoring the latter explanation is found in the observation that ATP inhibits the DPN reaction. A mixture of DPN and TPN isocitric dehydrogenases was detected in *A. niger* (440) and in *P. chrysogenum* (416).

The unexpected appearance of active citrate utilization in certain intestinal bacteria has been shown to complicate assays of coagulase. False positive reactions were obtained when Ca^{++} was liberated from citrated plasma when citrate was degraded by the bacteria (440a).

CO_2 fixation into dicarboxylic acids.—The reactions resulting in CO_2 fixation into dicarboxylic acids have been studied in great detail. Utter & his collaborators (441, 442, 443), separated two completely distinct systems from avian liver. One enzyme is the "malic enzyme" which was known to fix CO_2 reductively with pyruvate and TPNH to form *L*-malate (444). This enzyme acts as an oxalacetic carboxylase with an optimum at pH 5.4 and is stimulated in this reaction by TPN. The other enzyme was originally purified on the basis of CO_2 fixation into oxalacetic acid in the presence of ATP. It has now been shown to catalyze the reaction:

$$\text{Oxalacetate} + \text{ITP} \rightleftarrows \text{phosphopyruvate} + CO_2 + \text{IDP}, \quad \Delta F = +589 \text{ cal.} \qquad 15.$$

There is some question whether adenine nucleotides can replace the inosinic compounds less efficiently or whether all of the observed effects of adenine nucleotides are the result of catalytic amounts of inosinic nucleotides in the presence of nucleotide diphosphate kinase. The kinase was found to be present in excess in the purified enzyme used. Although the purified preparations of what is called oxalacetic carboxylase are free from "malic enzyme," they still contain significant amounts of lactic dehydrogenase, myokinase, enolase, and malic dehydrogenase. Therefore, it is not inconceivable that the complex reaction, involving CO_2 fixation and phosphate transfer, requires two en-

zymes. The over-all reaction has a pH optimum near 6, and like the malic enzyme and simple oxalacetic carboxylases, requires a metal (Mn^{++}). Utter & Kurahashi suggest that the decarboxylation of oxalacetate to form phosphoenolpyruvate is probably involved in the synthesis of carbohydrates by animal systems. This route of phosphopyruvate formation has the advantage of operating in a thermodynamically favorable reaction, as contrasted with the unfavorable direct transfer from ATP to pyruvate.

CO_2-fixing reactions in plant preparations are also complicated by multiplicity. Tchen & Vennesland (445) have found extracts of wheat germ to catalyze both fixation of CO_2 into oxalacetate during decarboxylation in the presence of ATP and Mg^{++} and the synthesis of oxalacetate from phosphopyruvate and CO_2, in addition to the previously described "malic enzyme" activity. The condensation of CO_2 and phosphopyruvate is catalyzed by preparations which no longer catalyze the exchange reaction with oxalacetate. The formation of oxalacetate is very similar to the reaction described by Bandurski & Greiner (446), who used extracts of spinach leaves. The phosphoenoloxalacetate intermediate postulated by Bandurski was eliminated by Vennesland *et al.* (447), who carried out the condensation in the presence of DPNH and D_2O. Since the preparation contained malic dehydrogenase, the oxalacetate formed was trapped as malate, which was isolated for D analysis. Since no stable D was found in the malate, it was concluded that no enoloxalacetate could have been produced as an intermediate.

The enzymes which add CO_2 to phosphopyruvate are associated with two functions: carboxylation and phosphate transfer. Although none of the enzymes has been obtained in a pure state, there is no evidence that more than one protein is involved in any of the reactions studied. If single enzymes carry out the over-all reaction, it is necessary to assume that they differ with respect to phosphate acceptors and that the enzyme which transfers to a nucleotide catalyzes a reversible reaction, whereas the enzyme which transfers only to water operates only in the direction of CO_2 fixation. These enzymes and the "malic enzyme" serve important functions in cell economy. They provide the only known mechanism for replenishing tricarboxylic acid cycle compounds, which must be done to replace the intermediates removed for syntheses. The nucleotide-requiring oxalacetic decarboxylase also provides a mechanism for the reverse process: introduction of organic acids into the glycolytic scheme, as proposed by Utter & Kurahashi. The further elucidation of the reaction mechanisms of this group of enzymes should prove of considerable interest.

Fumarase and aconitase. Fumarase has been demonstrated in extracts of *Paramecium caudatum* prepared in a Mickle disintegrater, although no oxidative reactions could be detected in the preparations studied (448). An original procedure was reported by Frieden, Bock & Alberty (449) to give crystals of fumarase identical with those obtained previously by Massey. The crystalline preparation is homogeneous in the ultracentrifuge and electrophoresis apparatus and has a molecular weight of 220,000. No spectrophotometric

evidence was found for an enzyme-substrate complex. In kinetic studies Alberty *et al.* (450) found that deviations occurred in Lineweaver-Burk plots with fumarate as substrate, indicating a stimulation by substrate in high concentration. Both K_m values and V_{max} vary markedly with both pH and with phosphate concentration. These observations were interpreted in terms of an enzyme-buffer complex with different catalytic properties from the enzyme alone. It is of interest to note that the Michaelis constants obtained under various conditions and the corresponding maximum velocities give values for the equilibrium constant calculated from the Haldane equation $K_{eq} = (V_1/V_2) \cdot (K_{m_2}/K_{m_1})$ which agree very well with the theoretical values. Massey & Alberty (451) attempted to evaluate the dissociation constants of the active form of fumarase. The effects of various anions (including substrate) on the activity introduced some uncertainty. A group with a pK below 5.7 (possibly a carboxyl) and one below 7.2 (possibly an imidazole) seem to be involved.

Aconitase was purified 24-fold from an extract of pig heart by Morrison (452), who believes his preparations to be 80 to 85 per cent pure. An electrophoretic fraction from the purified enzyme still catalyzes citrate formation from *cis*-aconitate and from isocitrate at the same relative rates as all cruder fractions, and the reaction with either substrate is activated markedly by the addition of ferrous ions and cysteine. While this is consistent with the viewpoint that a single enzyme is responsible for reactions with both citrate and isocitrate, the demonstration would have been more convincing if the assays used the reactions *cis*-aconitate→citrate and *cis*-aconitate→isocitrate, in which there would be no possibility of a single rate-limiting conversion. As in the case of fumarase, aconitase has been shown to be influenced markedly by ions, such as phosphate. Under given conditions K_m values for Fe^{++} and cysteine indicate the binding of both to the enzyme (453). It was suggested that one of each is bound to form an active enzyme.

Aconitase in rat liver was studied by Dickman & Speyer (454). They found that the enzyme within mitochondria behaved quite differently from the soluble enzyme with respect to relative activities with the three substrates at various pH values. They interpret their results as demonstrating a great effect of the mitochondrial membrane in determining observed rates. The soluble enzyme was found to have no affinity for any of the isomeric monomethyl esters of *cis*-aconitate. Both the soluble and mitochondrial aconitase preparations from liver require ferrous ions.

Nossal (455) has prepared extracts of yeast by means of an original mechanical disintegrater which disrupts cells in as little as 10 sec. With such preparations both aconitase and fumarase originally are found associated with particles, but both activities become solubilized with increasing disintegration time. Other Krebs' cycle enzymes are also liberated from particles by this procedure. The yeast particles seem to be very much like mitochondria, and, as in the studies of Dickman & Speyer, the elimination of a barrier by solubilization results in apparent net increases in the amounts of fumarase

and aco itase of yeast extracts. Fumarase was found by Kuff (456) to be associated with mitochondria in mouse liver and to be solubilized easily by sonic os illation.

Com etitive inhibition of aconitase by fluorocitrate has been established as a me hanism of fluoroacetate poisoning. Morrison & Peters (457) used synthetic fluorocitrate and found the inhibition to be even greater than that produced by enzymatically produced fluorocitrate. The increased inhibition is attributed to a more effective isomer present in the synthetic compound.

Related compounds.—Analogues of conventional metabolites have been shown to be related to conventional pathways, although the specific reactions are not known. With several strains of bacteria, Gray & Brooke (458) found that adaptation to tricarballylic and itaconic acids resulted in simultaneous adaptation to certain members of the tricarboxylic acid cycle. Unfortunately, only intact organism studies were reported, and it is not known whether the observations relate to entry of the analogues into the conventional scheme or whether adaptation is concerned with cell permeability. Another analogue, citramalic acid has been isolated from apple peel by Hulme (459). The mechanism of itaconic acid synthesis from glucose by *Aspergillus terreus* was studied by Bentley (460). The isotope distribution found was reported to confirm the proposal of Corzo & Tatum (461) that citric acid is a precursor of itaconic acid and indicated that in this mold citrate was formed via acetate.

PHYSIOLOGICAL STUDIES ON CARBOHYDRATE METABOLISM

The voluminous literature on the role of hormones in carbohydrate metabolism continued to increase during this year. Because of the bulk of this literature and its diverse nature, this subject has not been included in this review, except for a few papers which include information at the chemical, rather than the physiological, level.

Hormones.—A series of reports by Bornstein and collaborators has described the properties of a serum lipoprotein present in diabetic animals which inhibits glucose uptake by rat diaphragm (462, 463, 464). The production of the lipoprotein requires both growth hormone and cortisone (462). Similar lipoproteins were subsequently extracted from anterior pituitary, from liver, and from muscle and were found to inhibit glucose utilization by muscle extracts (464). The inhibition observed with intact muscle could be reversed, at least partially, by insulin, and the same was true for a similar inhibition of glutathione synthesis by liver slices from fasted rats (463). These observations were interpreted as supporting the hypothesis that insulin acts at the hexokinase level. In cell-free systems insulin gave variable, small effects, and with such systems neither the lipoprotein nor insulin effects have been demonstrated with assays for single enzymes (464).

An effect of diabetes on glucose phosphorylation was also shown by Renold, Hastings & Nesbitt (465). Studies with liver slices from alloxan-diabetic

rats incubated with isotopic sugars showed that whereas fructose was converted to glucose-6-phosphate at normal rates, glucose phosphorlyation was markedly decreased. In such slices fructose was eventually converted largely to free glucose, and little appeared in glycogen. The increased formation of free glucose indicates an enzymatic change different from one influencing glucose phosphorylation. Direct measurements of glucose-6-phosphatase by Ashmore, Hastings & Nesbitt (466) showed increases in the level of this enzyme in livers of fasted or alloxan-diabetic animals. An effect of insulin in returning levels of the phosphatase toward normal was observed when insulin was given to the intact animals, but not when it was added to slices.

Fructose utilization.—The ability of humans to utilize glucose was reported by Albanese *et al.* (467) to decrease with age, whereas fructose utilization remained relatively constant. Hill *et al.* (468) showed that rats on a fructose diet have a decreased glucose tolerance which was not influenced by insulin. This effect was related entirely to the ability of the liver to handle glucose and was interpreted as an indication that "glucokinase" of liver is an adaptive enzyme. Unfortunately, no direct determinations of this enzyme were reported, and it must be concluded only that the physiological effects observed here and in the experiments discussed in the previous section refer to the mechanism of glucose assimilation, which may or may not be limited by phosphorylation.

Experiments of Wick *et al.* (469) with eviscerated, nephrectomized rabbits indicated that peripheral tissues are unable to utilize fructose but depend on the liver for conversion of fructose to adequate metabolites. A simultaneous report to the contrary was made by Van Itallie *et al.* (470) who infused fructose into humans and found constant arterial-venous differences.

Sugar transport.—The mechanism of transport of sugar across cell membranes has been studied by several approaches. The fundamental nature of this process remains obscure, but work within the last year has produced a number of observations which must be considered in any theory of transfer across membranes. Louran (471) has studied intestinal absorption of glucose and found that hyperglycemia decreases the rate of absorption, and eventually, when high enough, stops it, but that the process is not reversed. Hele (472) attempted to relate absorption of various sugars from rat intestine to the rate of their phosphorylation by intestinal dispersions. Under arbitrary conditions the relative rates of phosphorylation and absorption of certain sugars correspond, and a measure of hexokinase shows the right order of magnitude to account for the absorption of carbohydrate ingested by the rat in a 24 hr. period. The conclusion that absorption involves phosphorylation cannot be accepted without reservations, however, in view of the alternatives discussed below and the fact the Hele's results indicate that the correlation between phosphorylation and absorption does not hold for all concentrations of sugars.

Experiments of Hestrin-Lerner & Shapiro (473, 474) indicate that glucose is not transported as such or as a phosphate ester but is converted to an un-

identified intermediate. In experiments with isolated loops of intestine it was found that glucose disappears from the inside much faster than from the outside of the loop and that a material accumulates in the tissue which does not appear to be carbohydrate. *In vivo* experiments showed that glucose absorbed from the intestine did not increase the concentration of sugar in portal blood. C^{14}-labeled glucose appeared in both blood and intestinal extracts in a form only partly fermentable by yeast. Lactic acid was found to account for no more than 1/4 of the glucose absorbed. Little of the labeled glucose was converted to CO_2, leaving the bulk of the label to be accounted for as an unknown material participating in intestinal absorption. Wilson (475) has also shown the formation of a lactate gradient across rat intestine when glucose was applied to the mucosal side. A mechanism of transport involving ions is implied by his experiments, which show a transfer of bicarbonate against a gradient from the serosal to the mucosal sides of ileum.

An original suggestion on the mechanism of transport was made by Keston (476). He has found mutarotase, the enzyme which catalyzes α-β interconversions of sugars in liver and kidney of various animals. Since the existence of a free aldehyde form had been demonstrated polarographically (477) [much smaller values for this form were reported by Los & Wiesner (478)], this or a similar form is involved by Keston as a "preferentially absorbed form." The existence of such a preferentially absorbed form in biological systems is deduced from a correlation between active transport and susceptibility to mutarotase. Animal mutarotase is inhibited by low concentrations of phlorizin. Some properties of mold mutarotase were described by Levy & Cook (479), who found the enzyme in preparations of glucose oxidase from *Penicillium notatum*, but not in preparations from certain other molds. Activity measured in an automatic recording polarimeter was found with D-glucose, lactose, maltose, D-xylose, and D-galactose. Slight activity was reported with mannose or glucosamine.

RECENT REVIEWS

During the past year many excellent reviews have appeared, which are invaluable for maintaining perspectives in rapidly developing branches of carbohydrate metabolism. In addition to those already cited, these include reviews on glycogen metabolism by Cori (480), hyaluronic acid by Gibian (481), transglycosidation by Kalckar (482), and synthesis of polysaccharides by Hassid (483) and by Stacey (484). Glycolysis has been reviewed by Stumpf (485) and alternate pathways by Cohen (255). Carbohydrate metabolism in photosynthesis is included in reviews by Havinga (486) and Holzer (487). The tricarboxylic acid cycle has been reviewed by Green & Nordmann (488), Krebs (489, Nobel Prize Address) (490), and Ochoa (491). The effects of hormones on carbohydrate metabolism has been discussed by Chaikoff (492) and by Stadie (493).

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METABOLISM OF AMINO ACIDS AND PROTEINS[1,2]

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In the past five years our interpretation of amino acid interrelationships has gained considerably from the field of microbiology. Especially valuable have been the contributions from isotope studies which enable one to trace the interconnections between the different reaction sequences by which amino acids are formed from nonnitrogenous intermediates. The whole field of amino acid metabolism is gradually taking shape and is steadily improving in contrast and in detail. This makes it possible to compare, in a preliminary way, the various networks of reaction circuits in different microorganisms with the corresponding though, incomplete, networks in higher organisms. In view of the wealth of experimental data accumulated it is desirable to attempt such a comparative integration, and the following section on amino acid metabolism represents an attempt in this direction. In order to maintain a reasonable coherence in the account the reviewer has deliberately extended this survey to comprise some earlier data which fall outside the actual period under review.

The section on protein metabolism is dominated by a discussion on protein synthesis, in which subject a somewhat heterogeneous collection of data has been assembled. The papers selected for review refer to energy transfer in peptide synthesis, amino acid incorporation into proteins, peptide rearrangements, the role of nucleic acids, etc. Even here it has been necessary to cast an occasional glance towards earlier work in order to maintain continuity in the text. The reviewer has refrained from entering into problems connected with the role of amino acids and proteins in nutrition, cancer, and clinical biochemistry, in the hopeful expectation that they will be treated in other chapters in this volume.

Amino Acid Metabolism

Of the methods, mainly isotope techniques and mutant studies, that have been employed for investigation of the formation and interrelation of amino acids the valuable variant worked out by Abelson *et al.* (1, 2, 3) has been quite successfully used in a series of further investigations of the amino acids in bacteria (4, 5). This method, denoted as the isotope competition method, is based on the important observation that an exogenous amino acid can in-

[1] The survey of the literature pertaining to this review was completed in October, 1954.

[2] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DNA for desoxyribonucleic acid; DPNH for diphosphorpyridine nucleotide (reduced form); RNA for ribonucleic acid.

hibit the endogenous formation of the same compound from various substrates; the inhibitory effect is furthermore carried over to the formation of amino acids metabolically related to the competing compounds. As a result of the simplicity of the procedure—involving cultivation of small amounts of microorganisms on an over-all C^{14} labelled compound as substrate, hydrolysis and paper-chromatographic separation of amino acids, and radiographic analysis of the chromatogram spots—the method enables a rapid determination of amino acid interrelationships. The detailed identification of related carbon structures of various amino acids has, however, still to be carried out by the conventional procedures. These involve isotope distribution analysis by degradation and localization of isotope content in different positions of the amino acid structures as derived from differentially labelled substrates.

A large proportion of isotope work in relation to amino acid metabolism has been done with labelled acetate as a substrate component. The results of isotope distribution analysis carried out on alanine, aspartic acid, and glutamic acid, isolated from *Torulopsis utilis* (6), *Saccaromyces* (7), *Neurospora* (8), and *Escherichia coli* (9), all indicate a definite parallelism between these organisms with regard to acetate utilization. Furthermore, the labelling patterns (as derived from labelled acetate) in these amino acids of microbial origin are the same as those obtained by isotope distribution analysis of alanine, aspartic acid, and glutamic acid from regenerating rat liver, as shown by Högström (10). In view of the frequent allusion to the isotope labelling patterns in alanine, aspartic acid, and glutamic acid—tacitly assumed to reflect the distribution of acetate carbon in pyruvate, oxaloacetate, and α-ketoglutarate—these patterns are recorded in Figure 1.

The above distribution patterns of acetate carbon obviously represent only a summary of what has been found in aerobic systems so far. In some ways they serve as standards against which the corresponding patterns of acetate distribution in other amino acids could be compared. It is noteworthy that, with very few exceptions, the labelling patterns for the several amino acids reveal marked similarities from organism to organism, thus indicating that the metabolic organizations by which amino acids are formed in aerobic systems possess many features in common. In this connection it should be recorded that the amino acid interrelationships found in aerobic systems have their counterpart in some anaerobic organisms. *Rhodospirillum rubrum* (11, 12) and *Clostridium kluyveri*, recently investigated by Tomlinson & Barker (13) and Tomlinson (14, 15), have been cultivated on labelled acetate and labelled carbon dioxide. By examining the labelling patterns of amino acids in relation to the standard patterns of distribution of acetate carbon and carbon dioxide carbon in alanine, aspartic acid, and glutamic acid, a close correspondence to aerobic systems has been observed. In comparison with the labelling patterns of alanine, aspartic acid, and glutamic acid recorded in Figure 1, the corresponding patterns of the same amino acids from anaerobic organisms show the distribution of acetate carbon and

carbon dioxide presented in Figure 2. The general impression to be drawn, especially when information from the fields of enzymology and biochemical genetics is pooled, is one of marked similarity in the metabolic networks of amino acid formation in different organisms, aerobic as well as anaerobic.

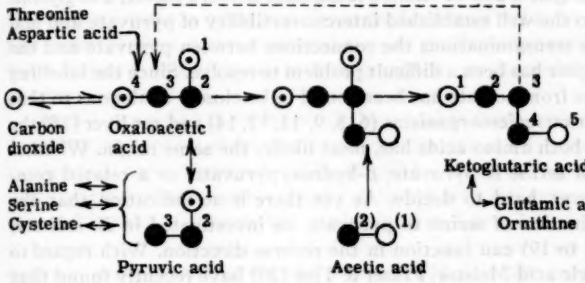
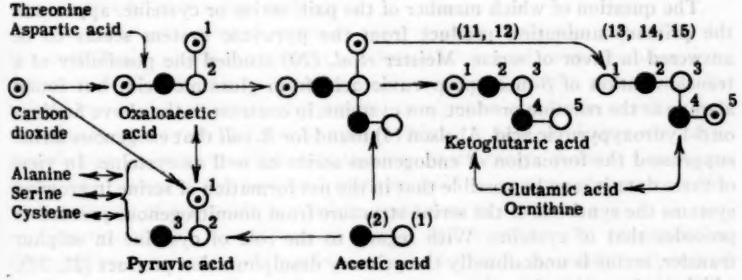


FIG. 1. Schematic representation of the distribution of acetate carbon in pyruvate, oxaloacetate, citrate, and ketoglutarate in aerobic systems, showing the corresponding distribution in related amino acids. The scheme is based on the results from investigation of aerobic microorganisms (6 to 9) and rat liver (10).



○acetate carboxyl carbon; ●acetate methyl carbon; ⊙recycling CO_2 (Fig. 1) or CO_2 used as carbon source by microorganisms (Fig. 2).

FIG. 2. Schematic representation of the distribution of acetate carbon, and carbon dioxide carbon, in anaerobic systems, represented by *Rhodospirillum rubrum* (11, 12) and *Clostridium kluyveri* (13, 14, 15). As in Figure 1, the distribution patterns are shown with regard to pyruvate, oxaloacetate, citrate, and ketoglutarate, and the amino acids related to these compounds.

True, the inherent restrictions in amino acid synthesis in heterotrophic organisms have to be taken into account; in some respects, however, these deviations from the "ideal" metabolic organization in autotrophs might be regarded as consequences of all-or-nothing effects. The real species-specific

divergences in the formation of one and the same amino acid are at any rate of rather rare occurrence. In view of this there may be some justification for the generalized treatment of the formation of amino acids, separate and in groups, which follows in the next section.

The pyruvate system and its relation to alanine, serine, cysteine, and glycine. —In contrast to the well established interconvertibility of pyruvate and alanine by various transaminations the connections between pyruvate and the serine-cysteine pair has been a difficult problem to resolve. Since the labelling pattern of serine from acetate has been found to be closely analogous to that of alanine in various microorganisms (6, 8, 9, 11, 12, 14) and rat liver (10) the C_3-structure of both amino acids has, most likely, the same origin. Whether the precursor of serine is pyruvate, β-hydroxypyruvate, or a related compound is, however, hard to decide. As yet there is no indication that the catabolic deamination of serine to pyruvate, as investigated in *E. coli* and *Neurospora* (16 to 19) can function in the reverse direction. With regard to β-hydroxypyruvic acid Meister, Fraser & Tice (20) have recently found that it can be transaminated, from glutamic acid in rat liver, to form serine. If the formation of β-hydroxypyruvic acid from glyceric acid by dehydrogenation could be established the connection of serine with pyruvic acid would be evident. An example of this reaction in the reverse direction has been reported [(32); see p. 279].

The question of which member of the pair, serine or cysteine, appears as the primary amination product from the pyruvate system seems to be answered in favor of serine. Meister *et al.* (20) studied the possibility of a transamination of β-mercaptopyruvic acid from glutamic acid, but found alanine as the reaction product, not cysteine, in contrast to the above findings on β-hydroxypyruvic acid. Abelson (4) found for *E. coli* that exogenous serine suppressed the formation of endogenous serine as well as cysteine. In view of these data it may be possible that in the net formation of serine in growing systems the synthesis of the serine structure from nonnitrogenous precursors precedes that of cysteine. With regard to the role of cysteine in sulphur transfer, serine is undoubtedly the primary desulphuration product (21, 22), which confuses the picture.

Another complication arises from the reactions involving the glycine-serine interconversion. In a number of microorganisms it has been shown that the isotope distribution, from labelled acetate, in carbon atoms 1 and 2 of glycine and serine is very similar (6, 9, 12, 14, 15), in full agreement with the view that serine could be the precursor of glycine. A further support for this idea is given by Abelson (4) who shows that exogenous serine supresses the endogenous formation of glycine in *Escherichia coli*. On the other hand, if glycine could be formed by a route not involving serine, the formylation of glycine to serine might provide a pathway of net synthesis of the latter amino acid. Olson (23) has recently found an enzyme system in many microorganisms, including bakers yeast, that catalyses a fission of isocitric acid to succinate and glyoxylic acid. Since the latter can be transaminated to gly-

cine, as shown by Tolbert & Cohan in green plants (24), this opens up new possibilities for glycine (and serine) biosynthesis. A fission of citrate and aconitate to succinate and glyoxylic acid has been observed by Campbell *et al.* (25). It should be mentioned that the expected isotope distribution (from labelled acetate) in serine and glycine would be in full accord with the exprimental findings, whether a direct conversion of pyruvate to serine→glycine is involved, or whether the pathway of synthesis involves the circuitous route from pyruvate via oxaloacetate, citrate, isocitrate, glyoxylic acid to glycine→serine.

In the conversion of serine to glycine another reaction sequence has been demonstrated, other than the plain deformylation of serine. Soloway & Stetten (26) and Sprinson *et al.* (27, 28, 29) have together furnished the proof that the ethanolamine part of serine is transferred intact to glycine via choline and betaine. Consequently carbon atoms 3 and 2 of serine might contribute to carbon atoms 1 and 2 of glycine. Taking into account the resynthesis of serine from glycine by formate addition, the functioning of the choline cycle will bring about a constant exchange of the carboxyl and the hydroxymethyl parts of serine, the α-carbon atom being intact. In Figure 3 an attempt has been made to summarize the complicated circuits involved in the formation and interconversion of serine and glycine, to which has been added the reaction sequence leading from aspartic acid via threonine to glycine (discussed in the next section).

The decarboxylation of serine to ethanolamine has a counterpart in the reactions leading from cysteine to taurine. With regard to mammalian liver Awapara & Wingo (30) and Eldjarn (31) have studied the compounds involved in the decarboxylation reaction; the former are of the opinion that cysteine is converted to 2-amino-ethane-sulphinic acid, and that this compound later undergoes oxidation to taurine. In the biosynthesis of Coenzyme A the formation of the aminoethanethiol residue involves, however, a decarboxylation of a peptide bonded cysteine (see p. 293).

Concerning the catabolism of serine and cysteine the nonoxidative deamination to pyruvic acid has been studied in detail, especially with regard to the participation of pyridoxal phosphate [Metzler & Snell (16, 17); Yanofsky (18, 19)]. A most interesting paper by Stafford, Magaldi & Vennesland (32) shows that β-hydroxypyruvic acid, related to serine, can be hydrogenated by $DPNH^2$ to D-glyceric acid in parsley leaves.

Glycine catabolism has been obscure, especially the postulated deamination to glyoxylic acid. Now however, Nakada & Weinhouse have definite proof of the presence of an enzyme system in rat liver and kidneys which metabolizes glycine to glyoxylic acid, glycollic acid, and formate (33). The total reaction sequence runs: Glycine→glyoxylic acid→glycollic acid→CO_2 +formate→CO_2.

Aspartic acid, threonine, methionine.—The indications that aspartic acid might provide the carbon skeleton of threonine and the homocysteine part of methionine emanated originally from observations of the analogous

labelling in threonine and aspartic acid from carboxy-labelled acetate. The organisms investigated were *T. utilis* and *E. coli* (6, 9); the same observation was later made by similar investigations on *Neurospora* (8) and the anaerobic *Rhodospirillum* (11, 12) and *C. kluyveri* (14, 15). Using the isotope-competition technique Abelson (4) could confirm the interconnection between aspartic acid and threonine in *E. coli*.

The actual mechanism involved in the reduction of the β-carboxyl of aspartic acid has recently been the subject of a series of investigations by Black *et al.* (34, 35). Investigating *E. coli* they found that aspartic acid, phosphorylated by ATP^2 to β-aspartyl-phosphate, became hydrogenated by DPNH to homoserine, a reaction that has been shown to be inhibited by glutamic acid in *Leuconostoc dextranicum* (36). Virtanen and his group have demonstrated the widespread occurrence of homoserine in the plant kingdom as a nonprotein constituent of plant cells (37, 38, 39).

The homoserine→threonine conversion, originally demonstrated by Teas *et al.* (40) in threonine-less *Neurospora* mutants, has been further studied in *Bacillus subtilis* (41) by Teas, and in *E. coli* under anaerobic conditions in the presence of glucose, by Cohen & Hirsch (42, 43). The reaction, which involves transfer of the hydroxy group in the gamma position in homoserine to the beta position in threonine, has been shown to be catalyzed by an ATP-dependent enzyme, as demonstrated in *in vitro* studies on bakers' yeast by Watanabe & Shimura (44).

Since the formation of homocysteine from homoserine is already established, the whole synthesis of methionine from aspartic acid now seems to be settled with regard to all intermediates. In a most interesting work Berg (45) has shown that in pigeon liver the methylation of homocysteine evidently proceeds via a compound with an —S—CH_2—OH-configuration, which probably is formed by formaldehyde addition, and is later dehydrogenated to —S—CH_3. If this turns out to be a general route for formation of methionine from homocysteine the origin of transferable methyl groups from formate, and ultimately from acetate methyl, could be regarded as solved. With regard to the further role of methionine as methyl donor, probably in the form of the S-adenyl derivative of Cantoni (46), some observations from the field of alkaloid chemistry indicate participation of methionine in the formation of the N-methyl in hordenine, and the N-O-methyls of ricinine [Kirkwood *et al.* (47, 48, 49)]. The formation of creatine from guanidoacetic acid via S-adenyl-methionine has been demonstrated by Cantoni & Vignos (50).

The catabolism of methionine most likely proceeds via demethylation to homocysteine, desulphuration, and conversion of the homoserine so formed to threonine. From this compound two separate branches seem to lead back to the nonnitrogenous metabolites. In mammalian organisms a fission of threonine has been observed, yielding acetate from the 3,4-part (51) and glycine from the 1,2-part of the molecule. That the formation of glycine from threonine proceeds without deamination has been most convincingly demonstrated by Sprinson and his group (51, 52). Another route for threonine

metabolism leads over α-ketobutyric acid (19, 53, 54), thus providing part of the structure of isoleucine (see page 286). It is possible that α-ketobutyric

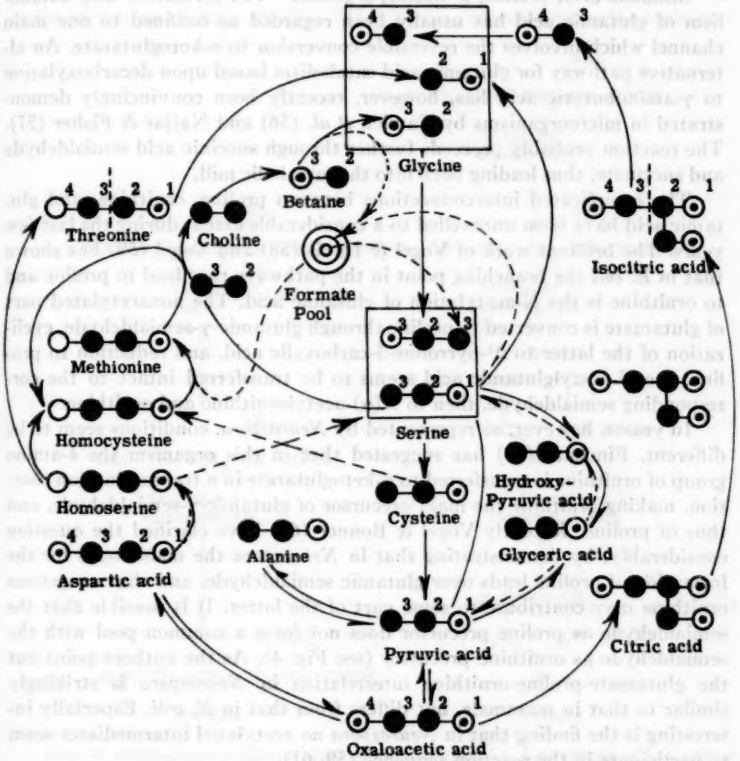


FIG. 3. Schematic representation of the interrelation between serine-glycine and other amino acids and metabolites. As a guide in judging isotope distribution patterns, the carbon atoms 3 and 2 of pyruvate are represented by filled rings. Carboxyls are denoted with ring and dot. The numeration of pyruvate and oxaloacetate gives the standard for judging the origin of glycine and serine carbon atoms from various sources. As seen the origin of glycine carbon atoms could be separated in at least three different cases. With regard to serine, recycling of the 3-2-fragment via the choline cycle and formate fixation to glycine returns a serine differently labelled as compared with the starting material. Three concentric rings represent the formate pool.

acid also plays a part in lysine synthesis in *E. coli* (4) and *Lactobacillus arabinosus* (55), since in these organisms aspartic acid has been indicated as a precursor of lysine.

The reactions involved in the metabolism of aspartic acid and related compounds are summarized in Figure 3. Much versatility is indicated.

Glutamic acid, proline, ornithine, arginine.—The formation and catabolism of glutamic acid has usually been regarded as confined to one main channel which involves the reversible conversion to α-ketoglutarate. An alternative pathway for glutamic acid catabolism based upon decarboxylation to γ-aminobutyric acid has, however, recently been convincingly demonstrated in microorganisms by Camien *et al.* (56) and Najjar & Fisher (57). The reaction probably proceeds further through succinic acid semialdehyde and succinate, thus leading back into the metabolic mill.

The complicated interconnections between proline, ornithine, and glutamic acid have been unravelled to a considerable extent during the last few years. The brilliant work of Vogel & Davis (58) and Vogel (59) has shown that in *E. coli* the branching point in the pathways that lead to proline and to ornithine is the N-acetylation of glutamic acid. The nonacetylated part of glutamate is converted to proline through glutamic-γ-semialdehyde, cyclization of the latter to Δ^1-pyrroline-5-carboxylic acid, and reduction to proline; the N-acetylglutamic acid seems to be transferred intact to the corresponding semialdehyde, then to N(α)-acetylornithine and ornithine.

In yeasts, however, as represented by *Neurospora*, conditions seem to be different. Fincham (60) has suggested that in this organism the δ-amino group of ornithine is transferred to α-ketoglutarate in a transamination reaction, making ornithine the main precursor of glutamic-γ-semialdehyde, and thus of proline. Recently Vogel & Bonner (61) have clarified the question considerably, by demonstrating that in *Neurospora* the main route for the formation of proline leads over glutamic semialdehyde, and that exogenous ornithine may contribute to some part of the latter. It is possible that the semialdehyde as proline precursor does not form a common pool with the semialdehyde as ornithine precursor (see Fig. 4). As the authors point out the glutamate-proline-ornithine interrelation in *Neurospora* is strikingly similar to that in mammals, but differs from that in *E. coli*. Especially interesting is the finding that in *Neurospora* no acetylated intermediates seem to participate in the reaction sequence (59, 61).

It seems that with regard to the formation of proline and ornithine from glutamic acid we have one of those rare instances where two protein constituents may be synthesized along two different reaction sequences, inherent in different groups of organisms, in this case represented by *E. coli* and *Neurospora*. The formation of lysine in the same organisms is another example (see p. 284). With regard to the formation of proline in mammalian organisms the situation awaits further elucidation with regard to the participation of glutamic acid. Proline appears to be formed directly from this substance or from ornithine through glutamic-γ-semialdehyde.

The formation of citrulline from ornithine seems still to involve a very complicated series of reactions. The thorough work of Grisolia & Cohen, carried out over many years, has established without doubt that the δ-carb-

amylation of ornithine is catalyzed by N-acyl-glutamic acids in the presence of ATP. In some later work Grisolia & Cohen (62, 63) have demonstrated that N-carbamyl-glutamic acid can be replaced by N-acetylglutamic acid in the formation of the labile CO_2-NH_3 complex that effectuates the carbamy-

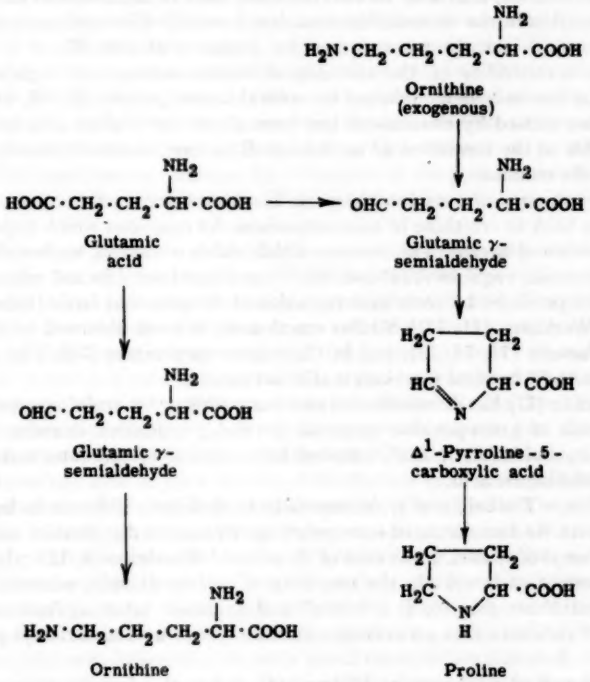


FIG. 4. Glutamate-proline-ornithine interrelation in *Neurospora*. Glutamic γ-semialdehyde is shown twice to emphasize the possibility that the semialdehyde as proline precursor does not form a common pool with the semialdehyde as ornithine precursor. (From Vogel, H. J., and Bonner, D. M., *Proc. Natl. Acad. Sci. U. S.*, **40**, 689, 1954.)

lation of the δ-amino part of ornithine. Recently Grisolia, Burris & Cohen (63a) demonstrated that in the formation of the complex intermediate the hydrogen atoms of the glutamyl portion of carbamyl-glutamic acid are not involved. In mammalian organisms, as demonstrated for rat liver, ammonia seems to be utilized as such in the formation of citrulline. In bacteria, represented by *Lactobacillus arabinosus*, the requirements for nitrogen seem to be more specific, and involve glutamine as the ammonia donor, as shown by Ory, Hood & Lyman (64).

The final amination of citrulline to arginine seems now definitely to be connected with arginosuccinate as an intermediate. The preparation of this compound by two different biosynthetic methods has been made by Ratner, Parker & Petrack (65), demonstrating that the compound easily forms a cyclic anhydride, that may be enzymatically split to arginine and fumarate. Its formation in the mammalian organism from citrulline and aspartic acid has been convincingly demonstrated by Ratner *et al.* (66, 67).

The reversibility of the splitting of arginosuccinate to arginine and fumarate has been demonstrated by several investigations (67, 68, 69). The inhibition caused by canavanine has been shown by Walker (70) to be attributable to the formation of an intermediate canavanino-succinate which blocks the reaction.

In recent years considerable work has been done on the conversion of arginine back to ornithine in microorganisms, by reactions which bypass the intervention of urease. The process, which yields ornithine, carbon dioxide, and ammonia, requires ATP and Mg^{++}, and has been effected with a cell-free juice prepared by sonic disintegration of *Streptococcus lactis* [Korzenovsky & Werkman (71, 72)]. Similar reactions have been observed in *Streptococcus faecalis* (73, 74, 75), and in *Clostridium perfringens* (76). The role of ATP or ADP[1] in these reactions is still not clear.

Nakada (77) has demonstrated another pathway for arginine catabolism in a strain of gram-positive bacteria, involving oxidative deamination to α-keto-δ-guanidovaleric acid, followed by oxidation of this intermediate to γ-guanidobutyric acid.

Lysine.—The origin of lysine seems to be definitely different in bacteria and yeasts. As demonstrated some years ago by isotope distribution analysis, the carboxyl of lysine, in the case of *E. coli* and *R. rubrum* (9, 12) originates from acetate carboxyl via the recycling of carbon dioxide, whereas in *T. utilis* and *Neurospora* (6, 8) a "clean" and dominant labelling from acetate carboxyl indicates that an acetate structure makes up the carboxyl part of lysine.

With regard to bacteria Abelson (4), using the isotope competition method, could confirm that in *E. coli* lysine seems to be related to aspartic acid, which agrees with the above observations concerning the equilibrium of the lysine carboxyl with recycling respiratory carbon dioxide. The nature of the precursors involved in the bacterial synthesis of lysine is still somewhat uncertain. At any rate α-aminoadipic acid is not involved, as shown by Davis (78); the intermediate role of α-ε-diaminopimelic acid is more likely (78). The latter acid, originally isolated from *Corynebacterium diphtheria* and *Mycobacterium tuberculosum* by Work (79), has later been isolated from several bacterial species (80, 81, 82). With regard to its alleged function as a lysine precursor Davis (78) has cautiously expressed the view that in organisms having a constitutional requirement for α-ε-diaminopimelic acid, this compound might provide the lysine structure. Presumably this reaction involves a decarboxylation, yet *L. arabinosus* seems to bring about the syn-

thesis of lysine from aspartic acid without the participation of diaminopimelic acid (55).

The conditions in yeasts, however, seem definitely to involve α-aminoadipic acid as the immediate lysine precursor (83, 84, 85). With regard to the formation of this intermediate Strassman & Weinhouse analyzed the isotope distribution in lysine from carboxyl-labelled acetate (86, 87) and found a dominant incorporation in the carboxyl and the C-6 atom. As an explanation they propose a condensation of succinyl-CoA[3] with acetate, or the keto group of ketoglutarate with acetate, followed by decarboxylation of the original α-carboxyl of the former acid. In both cases the carbon structure formed should have a labelling from carboxyl-labelled acetate in accordance with the experimental findings. By amination of the primary reaction product postulated by Strassman & Weinhouse α-aminoadipic acid should be formed.

Somewhere in the route leading from α-aminoadipic acid one might expect to find an α-amino adipic semialdehyde involved, free or acetylated, as a parallel to the formation of ornithine from glutamic acid. The isolation of piperidine-2-carboxylic acid from beans and from *Neurospora* by Lowy (88) points in this direction, this compound being most probably an analogue of proline in the glutamic acid series. It has also been isolated from a variety of microorganisms and plant material (37, 89 to 92). Neither this amino acid nor α-aminoadipic acid is an ordinary protein constituent, the only indication contrary to this view coming from Windsor (85) who finds bound α-aminoadipic acid in corn protein. Distribution of α-aminoadipic acid, as the free amino acid, has been investigated in plants by Berg *et al.* (39).

It is interesting to note that in higher organisms the catabolism of lysine leads through α-aminoadipic acid as an intermediate. The further metabolism of this intermediate involves glutaric acid, later converted to glutamic acid, as recently shown by Miller *et al.* (93, 94, 95). The mechanism involved in the conversion of α-aminoadipic acid to glutaric acid seems to be an oxidation of the alpha end of the molecule, with loss of the α-carboxyl as carbon dioxide. Some of the glutarate is metabolized in the rat to acetate and acetoacetate (96). By labelling lysine in the 6-position this atom was to a large extent recovered in glutamic acid-γ-carboxyl, a minor amount being split off as formate. In addition, part of the activity emanating from lysine-6-C^{14} appeared in the α-carboxyl of glutamic acid, thus confirming the assumption of a symmetrical intermediate. A certain difference seem to be apparent with regard to these reactions in rats and dogs (95).

Leucine.—The origin of leucine is somewhat obscure. That the carboxyl and the α-carbon are derived from acetate in a rather direct route was demonstrated some years ago for a number of microorganisms (6, 8, 9, 12). Recently Reed *et al.* (96a) were able to demonstrate the total distribution of acetate carbon in leucine from bakers' yeast. It was found that the total carbon structure of leucine, except the carboxyl, was derived from acetate methyl groups; the carboxyl, as expected, came from the acetate carboxyl.

A similar study of the distribution of pyruvate carbon gave a contribution of 25 per cent from pyruvate carbon atom 2 to each of the leucine carbons 3 and 4, plus a 50 per cent incorporation into the carboxyl. As a hypothesis the authors propose a route for the formation of leucine via a condensation of acetate by the methyl group to the keto group of α-ketoglutaric acid, followed by a double (oxidative) decarboxylation to 4-keto-*n*-valeric acid. This, in turn, might condense with another acetate unit in the same manner, yielding by decarboxylation and isomerization α-keto-*iso*-caproic acid; the latter as shown by Rudman & Meister (97) for *E. coli*, may transaminate with various amino acids to give leucine. In many respects this scheme looks reasonable, yet is hard to bring into accord with the view of Abelson (4) that α-keto-*iso*-valeric acid is involved as an intermediate in the biosynthesis of leucine in *E. coli*. More information on the subject is evidently required.

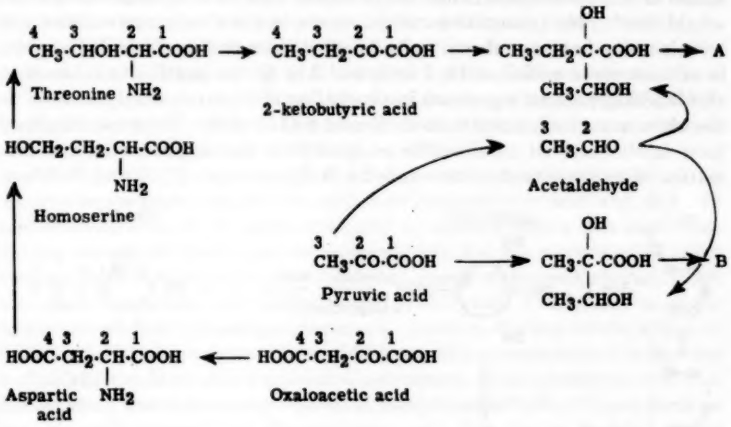
Valine, isoleucine.—Until recently there has been a considerable difficulty in obtaining a reasonable working hypothesis for the formation of these amino acids. The early finding that in valine, acetate methyl groups directly or indirectly contribute to the whole side-chain structure, (6, 8, 9) later confirmed by McManus (98), has been very hard to explain on the basis of our present knowledge of intermediary metabolism. Likewise, the finding that threonine in some way participates in the formation of isoleucine (4, 99) has been especially hard to bring into accord with the labelling, from acetate, in threonine and isoleucine and its precursors. Three recent papers by Adelberg (100), Strassman, Thomas & Weinhouse (101), and Strassman *et al.* (102) have, however, shown a way out of this dilemma.

With regard to valine, Strassman *et al.* propose an acetoin condensation of acetaldehyde and pyruvate as the first step, later followed by a pinacoline-rearrangement; this involves the migration of a methyl from position 2 to position 3. The resulting structure should be identical with the 2,3-dihydroxy-isobutyric acid known to be a precursor of valine (103). By the same reasoning the formation of the isoleucine structure should involve an acetoin condensation of acetaldehyde and 2-ketobutyric acid, followed by the migration of an ethyl group from position 2 to 3; this would yield the 2,3-dihydroxy-methylvaleric acid proposed as an isoleucine precursor (104) (see Fig. 5).

In the above scheme the results of Adelberg fit in nicely. If threonine is the donor of 2-ketobutyric acid (19, 53), it is to be expected that carbon atoms 1 and 2 of threonine will appear in positions 1 and 2 of isoleucine and that threonine carbons 3 and 4 should make up the ethyl-moiety of the tail-end of the isoleucine structure. The results of Adelberg with labelled threonine confirm, in a convincing way, the soundness of the scheme. Thus the question as to how valine and isoleucine are formed in yeasts and bacteria seems now to have a satisfactory explanation, in accordance with all available experimental data.

With regard to the catabolic fate of isoleucine Coon, Abrahamson & Green have shown that in liver the reaction proceeds through the keto

analogue of isoleucine and 2-methylbutyric acid, the latter undergoing a split to propionate and active acetate (105).



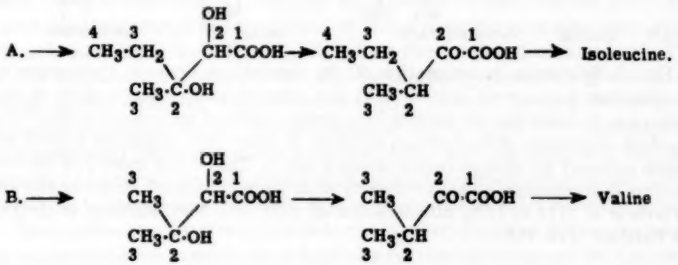


FIG. 5. Scheme of the formation of the carbon structures of isoleucine and valine according to Adelberg (100) and Strassman *et al.* (101, 102).

Phenylalanine, tyrosine, tryptophan.—The central problem with regard to the biogenesis of the aromatic amino acids has been the origin of the aromatic ring systems. On the basis of isotope distribution analysis on tyrosine from labelled acetate (106 to 109) and tyrosine and phenylalanine from labelled acetate and labelled glucose (110) Ehrensvärd & Reio (108) and Gilvarg & Bloch (110) concluded that in microbial biosynthesis of these amino acids glucose fragments were the starting material for the aromatic rings. The organisms investigated were *T. utilis, Neurospora, Saccaromyces,* and *E. coli.*

As intermediates in the synthesis, structures of seven carbon atoms related to heptoses were proposed (107, 108, 110). Assuming a C_3+C_4 condensation of glucose fragments one would expect that carboxyl-labelled acetate would label three consecutive carbon atoms in the C_7-structure. Since the tyrosine ring has been shown to be labelled from carboxyl-labelled acetate in at least three positions (4, 3 or 5, and 2 or 6), the possibility exists that this labelling pattern represents incorporation of acetate carboxyl carbon in the three consecutive positions 4, 5, and 6 (107, 109). These assumptions have lately received considerable support from investigations on the formation of the tryptophan structure by Rafelson *et al.* (111) and Rafelson

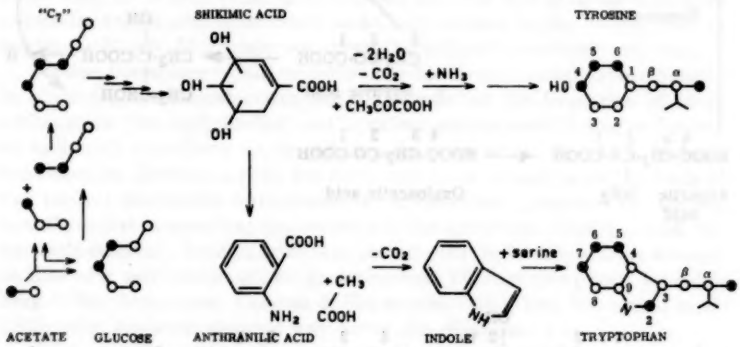


FIG. 6. Schematic representation of the connections between tryptophan and tyrosine-phenylalanine via shikimic acid. For comparison the carbon atoms of tryptophan and tyrosine that are derived from acetate carboxyl are denoted as black dots. The three ring-atoms of the benzene structure in these amino acids are derived from acetate carboxyl via glucose carbon atoms 3 and 4. The carbon atom 2 of the indol structure of tryptophan is derived directly from acetate carboxyl. Based on the work of Davis *et al.* (114 to 119), Ehrensvärd *et al.* (107, 108, 109), Rafelson *et al.* (111), and Rafelson (112, 113).

(112, 113). Analysis of the distribution of acetate carboxyl carbon in tryptophan, derived from *Aerobacter aerogenes*, showed that the three consecutive carbon atoms 7, 6, and 5 of the benzene part of tryptophan (see Fig. 6) were derived from acetate carboxyl (111) and from carbon atoms 3 and 4 of glucose. By dilution experiments with nonlabelled glucose in the presence of carboxyl-labelled acetate it was demonstrated that the appearance of acetate carboxyl carbon in the benzene ring of tyrosine involved an intermediary formation of glucose, in which acetate carboxyl entered in positions 3 and 4, as postulated earlier with regard to the labelling of tyrosine from acetate (107, 109). Thus the patterns of isotope distribution in tyrosine and tryptophan look reasonably analogous with regard to the distribution of acetate carbon and glucose carbon in the benzene ring systems (Fig. 6).

The above distribution patterns for tyrosine and tryptophan tell, however, very little with regard to the intermediates involved in the reaction sequence leading from glucose to the aromatic ring systems. Somewhere in this series the cyclic seven-carbon compounds: 5-dehydroquinic acid, 5-dehydroshikimic acid, and shikimic acid should be placed, their importance as precursors for aromatic ring systems being demonstrated by the brilliant investigations of Davis and his group (114 to 117). In a recent paper by Mitsuhashi & Davis the presence of enzymes in *E. coli* which catalyze the interconversion of the above acids has been demonstrated (118).

Although the proximate origin of 5-dehydroquinic acid is still obscure the reaction series from this acid through 5-dehydroshikimic acid and shikimic acid leads directly to phenylpyruvic acid as recently shown in a most convincing manner by Davis and co-workers (119). From a mutant of *E. coli* a very unstable compound was isolated, which easily underwent aromatization with decarboxylation, and yielded phenylpyruvic acid. The formation of the labile intermediate, named prephenic acid, seems to involve condensation of shikimic acid and pyruvate, the methyl group of the latter joined in position 1 of shikimic acid to give a quinoid C_{10}-structure. Since phenylpyruvic acid is most likely the immediate precursor of phenylalanine (120, 121) a connection between shikimic acid and phenylalanine, and thus also of tyrosine, seems to be definitely established.

With regard to the formation of the indole structure of tryptophan some progress can be reported. Tatum *et al.* have found evidence for the participation of shikimic acid in the formation of the anthranilic acid structure (122), although nothing is known with regard to the mechanisms involved. The further conversion of anthranilic acid to indole presumably involves an acetate condensation via the methyl group to position 1 of anthranilic acid, the carboxyl part of the acetate structure later joining the nitrogen atom of anthranilic acid [Rafelson *et al.*; Rafelson (111, 112)]. In some way the condensation of acetate to anthranilic acid, with decarboxylation of the latter, appears to be parallel to the formation of prephenic acid from shikimic acid.

The mechanism by which the side-chain of tryptophan is introduced by the indole-to-serine condensation has been further investigated by Tatum & Shemin (123); the participation of an unsaturated intermediate formed from serine, probably an aminoacrylic acid is indicated.

Some evidence has been provided for the biosynthesis of *p*-aminobenzoic acid. In a study of symbiotic interrelationships between different species of *Lactobacilli* Nurmikko (124) found evidence that *p*-aminobenzoic acid could originate from phenylalanine via *p*-aminophenylpyruvic acid, and *p*-aminophenylacetic acid. It follows that *p*-aminobenzoic acid should be regarded as a reaction product from phenylalanine, not a compound directly related to shikimic acid, like anthranilic acid.

As pointed out in a recent review of the field of aromatic biosynthesis (125) the whole picture of the formation of aromatic amino acids is beginning to be more comprehensible with shikimic acid in the central position. from

which the pathways to anthranilic acid→indole→tryptophan, and to phenylpyruvic acid→phenylalanine→tyrosine radiate. What remains is to fill the gap between glucose and the earlier members in the hydro-aromatic acid series. It is possible that investigations on the formation of simple aromatic compounds in the fungi could contribute to this work, many of these compounds belonging not only to the C_7-series, possibly related to shikimic acid, but involving also C_8- and other structures that could hardly be related to the shikimic acid series. The solution of the problem of the cyclization of glucose fragment may hold some surprises.

Turning to the catabolism of phenylalanine and tyrosine, the recent literature contributes to the role of ascorbic acid in the oxidative rupture of benzene rings, via *p*-hydroxy-phenylpyruvic acid→homogentisic acid→fumaryl-acetoacetate. The reaction that proceeds in animal tissues requires Fe^{++}-ions and phosphate, as reported by Schepartz (126). Ascorbic acid is thought to maintain the necessary amount of Fe-ions at the Fe^{++} stage; in beef-liver preparations the presence of ascorbic acid activates at least two steps of the reaction sequence [Williams & Sreenivasan (127, 128, 129)]. In view, however, of the recent investigations of the general problem of hydroxylation of aromatic compounds by Udenfriend *et al.* (130) and Brodie *et al.* (130a) the action of ascorbic acid may be more specific. Experimental evidence from these works indicates that a reaction product of ascorbic acid and hydrogen peroxide, formed in the presence of Fe^{++} and oxygen, could be the active principle, by which electrophilic hydroxy groups are transferred to the electronegative sites in aromatic and heterocyclic rings, thus bringing about the specific hydroxylations that are prerequisite for subsequent oxidative rupture of the ring systems.

The many branches of tryptophan catabolism continue to draw interest. The fission of the tryptophan molecule into indole, pyruvate and ammonia, occurring under certain conditions in *E. coli*, has been studied in detail by Gooder & Happold (131). Kynurinase has been purified by Jacoby & Bonner (132); the activity of the enzyme seems to be specific for the kynurenine structure, notwithstanding the presence of an acyl group on the nitrogen, or of an hydroxyl group in position 3. Another enzyme, kynurenine-formamidase, has been found in *Neurospora* by Jacoby (133); it catalyzes the hydrolytic split of the formyl group in N-formyl-kynurenine. The finer details of kynurinase action have been investigated by Miller & Adelberg (134). Contrary to previous assumptions they find that no transamination is involved as a preliminary to the split into anthranilic acid and alanine.

In riboflavine-deficient rats a marked increase in kynurenine and kynurenic acid has been observed earlier (135). Mason, investigating this question, further finds a considerable amount of anthranilic acid conjugates in the urine of riboflavine-deficient rats after administration of tryptophan (136). It is possible that riboflavine is involved in the conversion of kynurenine to 3-hydroxy-kynurenine.

Histidine.—Developments with regard to histidine metabolism have been

rapid in the last few years. The combined use of microorganism mutants and of isotope distribution analysis has established a close connection between the five-carbon structure in histidine and the sugar series. The work of Ames, Mitchell & Mitchell (137) agrees well with the earlier findings of Vogel, Davis & Mingioli (138) that the final sequence of reactions leading to histidine involves histidinol, preceded by the corresponding 2-keto- and the 2-3-dihydroxy analogues. The somewhat surprising finding that the carboxyl group of histidine from many microorganisms is mainly derived from the methyl group of acetate (6, 9, 12) might be explained on the basis that the five-carbon structure in histidinol and its precursors could be derived from a pentose structure. Levy & Coon (139) have made a thorough analysis of the isotope content in histidine formed in the presence of glucose-1-C^{14}. With respect to the five-carbon chain they find a dominant labelling in position 5, with a minor contribution to carbon atom 2, thus indicating a relation to the pentose system, yet involving some complications that are difficult to explain at present. An enzyme catalyzing the conversion of histidinol to histidine has been purified from dried cells of *E. coli* by Adams (140). The participation of DPNH is indicated.

Carbon atom 2 of the imidazole ring is clearly of formate origin (139, 141). In turn, this part of the histidine structure is a formate donor, and enters into the ethanolamine part of choline (presumably via serine-3), as shown by Sprinson & Rittenberg (142), and into the N-methyl groups of choline (via S-methyl of methionine?) as reported by Toporek *et al.* (143).

In histidine metabolism glutamic acid seems to be formed from the five-carbon structure along a pathway with some unexpected features. In microorganisms as well as in animal tissue the first step has been shown to be deamination of histidine to imidazolyl-acrylic acid, urocanic acid, the ring being left intact [Tabor *et al.* (144, 145); Mehler & Tabor (146)]. With regard to the further metabolism of urocanic acid to glutamic acid Borek & Waelsch have presented some evidence that this reaction sequence involves an opening up of the imidazole-ring under the formation of α-formamidino-glutaric acid, later to become hydrolyzed to glutamic acid (147). The isolation of the former intermediate from histidine in the presence of an enzyme extract from cat liver has been reported by the same authors (147a). On the other hand Suda and co-workers (147b) are in favor of N-formyl-glutamic acid as intermediate in microbial degradation of histidine. This intermediate could, however, not support growth for *L. arabinosus* on a glutamic acid-free medium (147).

A rigid proof for the origin of the glutamic acid structure from histidine has been furnished by Wolf (148) using isotope distribution analysis. His results show that carbon atoms 1, 2, 3, 4, and 5 of glutamic acid correspond to carbon atoms 5, 4, 3, 2, and 1 of histidine; the N_1 of the imidazole structure becomes the amino group of glutamic acid.

A quite different pathway for histidine catabolism has been demonstrated in mussel tissue by Thoai *et al.* (148a) involving a successive shortening of

the side-chain with the ring intact. The principal products seem to be imidazolyl-acetic acid, imidazolyl-aldehyde, and imidazolyl-carbinol.

3-Methylhistidine has been identified as a normal constituent in human urine (149). Its origin is unknown.

Nonoxidative decarboxylation.—Of the reactions involved in the synthesis and catabolic utilization of amino acids nonoxidative decarboxylations apparently play a minor role, quantitatively. However, most or many of the naturally occurring amines are of great physiological importance so their relationships with the amino acids are obviously of considerable interest. Contributions to this field have been made by Schayer *et al.* (150, 151), who have shown that exogenous histidine is not the direct precursor for bound histamine in mammals. In this connection the same investigators report that the further metabolism of histamine involves the same diaminooxidase that attacks cadaverine (152). In view of the importance of aromatic amines in alkaloid synthesis it is interesting to note that the origin of tyramine and hordenine in plants has been confirmed by isotope studies (153, 154), likewise the formation of gramine from tryptophan (155).

Nitrogen transfer.—In an extensive study on nitrogen assimilation and protein synthesis in yeast Miettinen (156) has surveyed the scope of transamination reactions in general. He emphasizes that

> since 1950 a number of workers using the specific and sensitive paper chromatography method have shown on the one hand the existence of transamination reactions of α-ketoglutaric acid with practically any protein amino acid (and a number of non-protein amino acids), and on the other hand the existence of reactions involving neither α-ketoglutaric acid nor glutamic acid.

True, the list of both series of reactions is steadily increasing. In so far as glutamic acid is concerned an investigation of Rudman & Meister reports two groups of transaminases, A and B, one concerned with the aromatic amino acids, the other with valine, isoleucine, and leucine (97). The transamination which involves kynurenine in *Pseudomonas* has been studied by Miller, Tsuchida & Adelberg (157); furthermore the nitrogen transfer from δ-aminovaleric acid and α,γ-diaminobutyric acid has been studied in brain homogenates by Roberts (158).

In the series not involving glutamic acid Awapara & Seale (159) found that pyruvate could function as nitrogen acceptor for valine, isoleucine, leucine, methionine, and phenylalanine in various organs of the rat. The same type of system seems to work in *Brucella abortus* as reported by Altenberg & Housewright (160). In an extensive series of investigations Meister *et al.* (161 to 164) have studied the transaminase reactions in which the α-nitrogen of asparagine and glutamine are involved. In these reactions, the amide nitrogen is hydrolyzed off as ammonia. An interesting example of transamidation has been given by Scheffner & Grabow, who report a nitrogen transfer in bakers' yeast between glutamine and asparagine (165).

The coupled deaminations, known as the Stickland reaction, in which one amino acid can act as the donor, the other as acceptor of hydrogen, has been

the subject of a review by Nisman (166). This type of reaction seems to be an important mechanism for energy generation in many bacteria growing on amino acids as the sole source of carbon and nitrogen. The review of Nisman covers 90 references and is a very stimulating guide into this difficult section of amino acid metabolism.

With regard to the actual mechanism involved in transamination the role of metal ions in connection with the action of pyridoxal has been studied in model systems in the absence of any enzymes by Metzler & Snell (16) and Baddiley (167). In compiling this and other evidence for the formation of chelated structures in transamination reactions, Calvin (168) has visualized the structures involved as the result of one pyridoxamine-keto acid and one pyridoxal-amino acid mutually chelating at a central metal ion, through the two phenolic hydroxyls present and the amino nitrogens from the amino acid and pyridoxamine. As for the exchange of hydrogen atoms in the transamination reaction between aspartate and ketoglutarate Hilton *et al.* (169) have studied the reaction *in vitro* in the presence of D_2O. It was found that about two of the aspartate hydrogens were replaced by deuterium and in the glutamic acid formed the ratio glutamate:deuterium was 1:1. In view of these results reference should be made to the work of Kögl & deFlines (170) and Brandenberger & Cohen (171) concerning the alleged role of intermediate α,α'-imino-di-acids in transamination reactions.

In the introduction to this section it was mentioned that the anabolic side of amino acid metabolism seems to comprise networks of reactions that look rather similar in different organisms notwithstanding the fact that some branches of the circuits are lacking in heterotrophic organisms. With regard to the catabolic utilization of amino acids, however, these networks give the impression of comprising a variety of ramifications which depend upon the inherent biochemical individuality of the cells, organs, or organisms concerned. The species-specific distribution of nonprotein amino acids of various types in plants and animals, and the whole set of biogenous amines related to amino acids belong to this province, like the numerous pigments and alkaloids produced from aromatic acids by species-specific condensations of all kinds. Among all these reactions involving partial utilization of amino acids some, obviously, are characteristic for large groups of organisms, like the role of orthoquinones from tyrosine in crosslinking the protein of the insect cuticle; others are of omnicellular importance like the formation of nicotinic acid from tryptophan.

The latest example of the participation of amino acids in the formation of important biocatalysts is the synthesis of CoA, which has recently been the subject of a series of interesting investigations by Lewintow & Novelli (172), Hoagland & Novelli (173), and Brown & Snell (174). The pantoate part of the CoA seems to originate from valine by β-hydroxylation and exchange of the amino group for hydroxyl [Purko, Nelson & Woods (175)]. The next step should be condensation with β-alanine, as studied by Maas (176) and Maas & Novelli (177), followed by condensation with cysteine

(172, 173). Decarboxylation of the compound to pantetheine, phosphorylation, and combination with the adenyl moiety completes the CoA structure. The different steps in the assemblage of CoA illustrates the fact that amino acids have a variety of active functions in the living cell besides their mere passive, and temporary, existence as protein constituents.

Protein Metabolism

The notable advances that have been made in the last few years as regards protein structure are undoubtedly a challenge to the workers in the field of protein synthesis. The presentation of evidence for strict spiral arrangements in protein architecture by Pauling and his school (178, 179, 180) and the demonstration of specific sequences of amino acid units in peptide chains by Sanger & Tuppy (181) and Tuppy & Bodo (182) are decidedly a guide to our efforts to reveal the intricacies of protein synthesis and the mechanisms involved. Needless to say, the demands of an acceptable theory for protein synthesis are overwhelming, in as much as it should weave together our present notions on the energetics behind the formation of carbon-to-nitrogen linkages in amides, in peptides, and in polypeptides, on the arrangement of amino acid units in individual sequences, on the role of nucleic acids, and on the spiralization of polycondensates to specific structures of finite molecular weight. In our efforts to realize this ambitious program the value of competent compilations of data related to the various stages and mechanisms involved in protein synthesis cannot be sufficiently appreciated. With regard to the present report on the progress in this field it is with feelings of sincere gratitude that the reviewer can refer to a series of outstanding monographs appearing in 1953 and 1954, which cover some 2500 papers related to the subject.

Among these reviews and monographs there is one by Borsook on peptide bond formation (183), by Bricas & Fromageot on naturally occurring peptides (184), by Putnam on bacteriophages (185), and by Gale on assimilation of amino acids by Gram positive bacteria (186). Part A of the second volume of The Proteins contains, among a series of most interesting articles, a stimulating chapter on protein metabolism in plants by Steward & Thompson (187). A book "Mechanisms of Enzyme Action," has appeared, which contains many contributions directly related to the problem of protein synthesis, of which an article by Lipmann (188) is most inspiring. A clear and compact treatise on protein metabolism by Fisher (189) has been published and is very useful as a general introduction to the field.

In the following survey on protein metabolism, restricted to some special topics related to protein synthesis, the competent treatises of the above authors have been frequently consulted.

The formation of the amide linkage and the energy transfer involved.—The enzymatic formation of amino acid amides and lower peptides *in vivo* and *in vitro* has been the subject of considerable study since the first unequivocal demonstration of the participation of ATP in the formation of glutamine

(190, 191, 192). At present, the action of ATP and CoA in amide-bond synthesis in microorganisms, higher plants, and animal tissues presents a picture of considerable interest, and exerts a definite feed-back influence on our interpretation of amino acid polycondensation in general.

The ATP-dependent glutamine synthetase has been isolated and considerably purified by Elliott (193, 194), the presence of this enzyme in plants as well as in animal tissues being demonstrated (195). An enzyme preparation from sheep brain has been shown to be able to produce the γ-amide of 2-methylglutamic acid, as well as glutamine, the methylated glutamic acid being an inhibitor for glutaminase (196). In the synthesis of glutamine no phosphorylated intermediate has been identified, although an analogue to β-aspartyl-phosphate, demonstrated by Black *et al.* (34, 35), might be expected. This compound, an intermediate in homoserine synthesis, might well be aminolyzed to asparagine (34), yet the question awaits further investigation.

In the synthesis of hippuric acid, CoA is clearly involved, as demonstrated by Chantrenne (197) for liver tissue. As a model experiment Schacter & Taggart (198) show that an easy condensation of benzoyl-CoA and glycine can take place, independent of ATP, in the presence of an enzyme extract from liver. Interesting comments on this reaction have been made by Schwytzer & Hürlimann (199). In the synthesis of *p*-aminohippuric acid some indications point to the participation of CoA, as mentioned by Borsook (183).

Turning to tripeptides, the biosynthesis of pantothenic acid is clearly ATP-dependent, as shown by Maas (176) and Maas & Novelli (177), yet independent of CoA. The two-step condensation of the components in glutathione, as revealed by Bloch and his group (200), has been further studied by Yanari, Snoke & Bloch (201, 202). The enzyme which catalyzes the condensation of γ-glutamylcysteine and glycine has been considerably purified, and the dependence of the reaction on ATP, Mg^{++}, and K^{+} has been confirmed. As shown earlier (200) CoA is not involved. Webster (203), in an interesting study of glutathione synthesis in homogenates of bean hypocotyledons, confirms the results of Bloch on liver preparations. Even here CoA does not stimulate the rate of synthesis, yet, in addition to ATP, Mg^{++}, and inorganic phosphate, free SH groups in the enzymes involved seem to be necessary. Neither vitamin B_{12} nor folic acid affects the incorporation of glycine-2-C^{14} and cysteine-S^{35} into glutathione (204).

It is hard to visualize from the above data any mechanism in common for amide-bond formation in general. Recently, however, Lipmann (188) has made a very interesting proposition, based on the data from pantothenic acid synthesis (176, 177). The first stage of this synthesis is regarded as a pyrophosphorylation of the enzyme via ATP. Then the sites holding pyrophosphate groups are assumed to react with the carboxyl of pantoic acid, thus fixing the latter on the enzyme frame with liberation of pyrophosphate. The bond between the enzyme and pantoic acid should hold sufficient energy to

be easily aminolyzed, in this case with β-alanine. As Lipmann points out "the catalysis of pyrophosphate exchange by pantoate, but not by β-alanine, shows that carboxyl activation initiates peptide bond formation" (188). The reaction proposed could be written:

$$\begin{aligned} E + Ad{-}P \sim PP &\rightleftarrows E \sim PP + Ad{-}P \\ E \sim PP + R{-}COOH &\rightleftarrows E \sim COR + PP \\ E \sim COR + NH_2R' &\rightleftarrows E + ROC{-}NHR' \end{aligned} \qquad (A)$$

A counterpart to this phosphorylation at the enzyme level is the process involving "adenylization" of an enzyme, a reaction proposed by Lipmann (188) and Jones *et al.* (205) for the formation of acetyl-CoA from acetate and ATP.

$$\begin{aligned} E + Ad{-}P \sim PP &\rightleftarrows E \sim P{-}Ad + PP \\ E \sim P{-}Ad + HS{-}CoA &\rightleftarrows E \sim S{-}CoA + Ad{-}P \\ E \sim S{-}CoA + R{-}COOH &\rightleftarrows E + CoA{-}S{-}COR \end{aligned} \qquad (B)$$

Recalling the CoA-influenced synthesis of hippuric acid, this type of reaction leading to peptide bond formation might belong to series (B) of the above reactions, as a consequence of aminolysis by any amino acids of the thiol-ester formed. With regard to the CoA-independent formation of asparagine, glutamine and glutathione, reaction (A) would be the most probable.

Phosphorylation at the enzyme level, a prestage in protein synthesis?—As a logical extension of the hypothesis for the formation of pantothenic acid according to reaction (A) Lipmann (188) proposed a model for amino acid polycondensation which requires linear units of an unspecified kind (although polynucleotides might well apply) being pyrophosphorylated in successive centers at distinct distances from each other. To this frame, amino acids are assembled, condensing with the carboxyl groups with pyrophosphate liberation. The next step would be condensation, head-to-tail, of the amino units fixed on the frame, whereby a peptide chain should be continually loosening from the enzyme surface.

A similar model has been presented by Dounce (206) in which amino acid phosphate esters are assumed to condense with a phosphorylated polynucleotide. In view, however, of the fact that phosphorylated intermediates have rarely been encountered in the formation of low-molecular peptides the Lipmann model looks more attractive as it involves the assemblage of amino acids as such. An interesting feature, common to both theories, is the assumption that from the primary assemblage of amino acids to the enzyme to the final stage, when the finished peptide is cleaved off, no intermediates are supposed to leave the enzyme surface. Remotely, this attractive idea reminds one of the hypothesis proposed by Lardy for the formation of long-chain fatty acids from acetate units: successive incorporation on an enzyme surface and retention there until the final product is split off (207). In this connection a series of interesting observations by Miettinen should be men-

tioned (156). Comparing low-nitrogen yeast with the same yeast under rapid cell-division Miettinen observed a marked difference in the ratio of ethanol-extractable and trichloracetic acid-extractable amino acids, the latter rapidly increasing in proportion to the rate of cell division. As a hypothesis, Miettinen regarded the acid-soluble amino acids as being displaced from a high molecular structure, possibly of polynucleotide nature. It is possible that the findings of Miettinen have a direct bearing on the mechanisms for amino acid assemblage, prior to polycondensation, as proposed by Lipmann and Dounce.

The question of specific incorporation of amino acids and the nature of "pool" components.—In view of the specific sequences of amino acid units in peptides isolated from various proteins there must be some stage in protein synthesis in which a certain selection takes place of the amino acids which condense to peptides. Lipmann (188) as a hypothesis, endows the enzyme(s) involved with a selectivity, and assumes that "amino-acid-specific-activation spots are lined up on a structure in the demanded sequence." From this point of view, the responsibility for the specific amino acid sequences in proteins lies in the specific structure of the enzyme, not in the local distribution of amino acids, free or in various combinations, at the loci of protein synthesis. From a purely chemical point of view it is hard to visualize such a degree of high specificity of any enzyme pyrophosphates, in which any carboxyl-holding compound might well compete with any amino acid for a certain site on the enzyme. Yet, we have to face the fact that specificity in the formation of protein structures of all kinds is largely (or wholly?) independent of the factors that influence the rate of protein synthesis. The difficulty is to keep these factors apart when analyzing any case of protein formation *in vivo* or *in vitro*.

With regard to the mechanisms involved in the formation of a few, well defined, proteins this question has elicited considerable interest in recent years. The unequal distribution of labelled amino acids in the ovalbumin molecule formed *in vivo* or *in vitro*, as reported by Steinberg & Anfinsen (208), has evoked a lively discussion. In a later work, Anfinsen & Flavin (209) find that ovalbumin, formed in the presence of labelled alanine, serine, glycine, and glutamic acid added to the medium, shows marked differences in the specific activities of these amino acids in different positions in the molecule. Likewise, insulin and ribonuclease show the same type of unequal distribution as regards labelled glycine and phenylalanine. Askonas, Campbell & Work (210), on the other hand, report a less pronounced unequal distribution from labelled amino acids in casein and β-lactoglobulin formed *in vivo*, in agreement with analogous results of Muir, Neuberger, & Perrone (211). Simpson & Velick (212) find no indication of unequal distribution of radioactivity in glycine, alanine, lysine, phenylalanine, and methionine in rabbit aldolase and glyceraldehyde phosphate dehydrogenase after a single injection of these labelled amino acids.

In a recent report Steinberg (213) summarizes the situation and remarks

that there may be a difference in the intracellular conditions in the *in vitro* systems investigated and in those cases where a certain protein has been produced under natural condition. This difference might lie in a certain sluggishness in the *in-vitro* systems, by which a heterogeneity of pool constituents could be retained for a longer time than in the rapidly operating *in-vivo* systems. As a matter of fact, the degree of non uniformity of labelling in the Steinberg-Anfinsen experiments becomes less striking after a few hours incubation, and approaches unity after 10 hr. The heterogeneity mentioned may involve the presence of amino acids in different combinations at the loci of protein synthesis. Miettinen (156) in a discussion on this subject remarks, with a glance at the template theory, "that exogenous (labelled) amino acids would be more easily available for some parts of the template, other parts utilizing preferentially amino acids synthesized *in situ*, or split from preformed amino acid derivatives." In turn, this point of view recalls the preferential utilization of exogenous amino acids in microorganisms, as observed by Abelson *et al.* (1 to 4) and McQuillen & Roberts (5).

It may well be that our simplified notions of "pool" amino acids at the loci of protein synthesis have to undergo some revision. In some cases, for example, the formation of adaptive enzymes in bacteria, free amino acids may be the actual units from which these specific proteins are formed, as concluded by Halvorsen & Spiegelman (214, 215, 216) and Monod *et al.* (217). In other cases the primary units to be incorporated may involve amino acids in combinations of various kinds; the influence of lower peptides on protein synthesis (see next section) might well have a bearing on this question, like the intricate systems in transpeptidation.

In addition, some work from the field of embryology points to still other pool components. Kavanau (218) in a study of protein metabolism in sea-urchin development, proposes that some low-molecular intermediates that seem to be present (keto acids, and peptides being excluded) are easily converted to amino acids by the sea-urchin embryo. However, despite what is known regarding the pool constituents, the attainment of protein specificity has still to be explained. The assumptions of Lippman (188) that the sole responsibility rests with the enzyme, largely independent of the composition of the medium, may not be too far off the mark. From this point of view the nature of the primary units, amino acids free or bound, that enter into combination with the catalyzing molecules prior to polycondensation would influence the rate of incorporation rather than its specific features.

The role of peptides in protein synthesis.—In a review of protein metabolism in bacteria Gale (186) remarks that the "formation of simple peptides cannot be taken as a model for protein synthesis," pointing to the insensitiveness of the formation of extracellular peptides in *Staphylococci* towards chloramphenicol, and the similar situation with regard to glutathione synthesis in *E. coli*. The same opinion has been expressed by Linderström-Lang (219) who proposes that transpeptidation reactions are stimulatory, rather than involving operating units in the polycondensations leading to proteins.

Yet the transpeptidations which center around glutathione are at any rate a most versatile set of reactions. The transfer of γ-glutamyl residues from glutathione to various amino acids has been the subject of detailed study by Hanes *et al.* (220), Fodor *et al.* (221, 222), and Hird & Springell (223). An interesting feature of these reactions is the marked specificity of the transferase enzymes toward α-amino structures of the amino acids involved, and a certain selectivity with regard to the transfer of γ-glutamyl groups to amino acids of different side-chain structure. As regards the role of these reactions, Steward & Miller (in 187, p. 587) have proposed that the various γ-glutamyl peptides could be the primary units presented to an enzyme surface at the early stages of protein synthesis: another contribution to the "pool" components. In view of the special properties of the γ-glutamyl linkage the suggestion offers interesting possibilities.

The stimulatory influence of peptides in general for protein synthesis (usually measured in terms of growth) is a very difficult question which involves the complex action of strepogenins. The field has been thoroughly reviewed by Bricas & Fromageot (184). In view of the difficulties involved in analyzing the mechanisms of peptide influence on the growth rate of living cells Snell and his group have carried out a pioneer investigation of great importance (224 to 229). Their results indicate that many of the cases where in peptides are found to be growth-stimulating are attributable to an altered uptake of certain amino acids from the medium, whereby inhibitory effects caused by some amino acids at certain concentrations are overcome. It seems to be very hard to formulate any definite rules for the peptide effects; they vary considerably, both qualitatively and quantitatively, for different peptides and organisms. In one paper (228) it is remarked that the most active peptides (histidine peptides acting on *L. delbruecki*) contain amino acids which do not occur in proteins—a fact that indicates rather conclusively that these peptides are not incorporated into the proteins of the test organism. Among other contributions to peptide effects on bacterial growth could be mentioned some studies on the *Pleuropneumonia* group (230), *Fusobacteria* (231), *Clostridium Feseri* (232) and on the *Leuconostoc* group (233). The utilization of phosphopeptones by *Lactobacilli* has been investigated by Ågren (234) and the use of *Lactobacillus bifidus* for strepogenin determination has been studied by Roine *et al.* (235). With regard to higher organisms an investigation of Schwarz (236) pertains to the effect of partial hydrolysates of casein on necrotic degeneration of the liver in dietary deficiencies.

Of special interest are the reports of the appearance of peptides with strepogenin-like effects in eggs and embryonic tissue at different stages of development. Gustafson *et al.* (237) found that, in some fractions of material from eggs and embryos of sea-urchins, compounds are present that, judged from their growth-stimulating effects on *Lactobacillus casei*, could be classified as peptides with strepogenin-like action. The variations in effect of these compounds are clearly correlated with the qualitative and quantitative changes in free amino acids in the embryos during the first 30 hr. of develop-

ment (238, 239). Similar observations have been made by Snellman & Danielsson in a study of the nitrogenous compounds in ripening peas, especially with regard to plant globulin formation (240). The presence of growth-stimulating compounds in extracts from animal embryos is an old observation for tissues cultured *in vitro*. Rosenberg & Kirk (241) and Gerarde & Jones (242) relate these effects to dialyzable peptides formed in animal embryonic tissue at early stages of development.

In summary, the present evidence for the participation of peptides in protein synthesis points mostly to an interference with the balance of free amino acids at the loci of amino acid assembly to proteins. In some cases an inhibitory effect, caused by a temporary dominance of one amino acid, may be relieved by peptides; in other cases a continual destruction of an amino acid (by oxidative deamination, for example) may be bypassed by the local administration of the same amino acid in peptide combination. If we now begin to see some light in this complicated display of competition effects it is largely thanks to the work of Snell *et al.* already mentioned (224 to 229).

The direct participation of peptides as intermediates in "growing" peptide chains has been proposed and discussed many times in connection with transpeptidation reactions. Linderström-Lang (219) has emphasized that rather specific requirements are needed for giving a peptide of moderate size a chance to become extended by dismutation into longer structures and shorter fragments. Yet this view appears in the work of Snellman & Danielsson (240) and of Danielsson (243) as a possible explanation for the correlation of peptide production in plants with the different phases of globulin production. It may be that the conditions in eggs, seeds, and embryos, where a large amount of preformed protein is utilized for production of other proteins, are different from those which would prevail in microorganisms, in which exogenous sources, nitrogenous and nonnitrogenous, would be mainly utilized. A decision cannot be made at present, whether the peptides observed at various stages of embryonal development represent fragments from yolk protein on their way to be incorporated as such into newly formed proteins, or whether they are synthesized *de novo* from amino acids. They may also be regarded as various catabolic products, later to be hydrolyzed and reutilized. As Borsook puts it in a review of the subject (183) "more data, and new kinds of data appear to be needed."

The role of nucleic acids.—In recent years there has been a considerable amount of effort expended in investigations of the alleged synchronism between nucleic acid formation and protein synthesis. In some ways, the results demand a revision of the earlier, somewhat dogmatic, view that an increase in nucleic acid content under rapid cell-division may be directly correlated with an increase in the protein content. The selective inhibition of one of the two processes [summary by Gale (186)] has evoked many explanations of all kinds. As an illustration of the prompt action of inhibitors like chloramphenicol, a recent work of Wisseman *et al.* (244) shows that the incorporation of glycine-1-C^{14} into the proteins of *E. coli* is completely in-

hibited under conditions wherein incorporation of the glycine in adenine and guanine of nucleic acids was still proceeding.

In a detailed study of the interrelation of nucleic acid and protein synthesis in the same organism Cavanna, Chain, Hedén, Malmgren, and the Hammarsten group (245) report an inhibitory action of metal ions on the turnover rate of DNA[3] and protein, but with RNA left unaffected.

The work of Cavanna *et al.* (245) is of considerable interest in view of detailed analyses of the rate of N^{15}-incorporation into nucleic acids and protein, in relation to the amounts of RNA[3], DNA, and protein at various stages of the lag phase and the logarithmic phase of growth. The results of these large-scale experiments (1000 liters of medium) indicate that the amounts of RNA, DNA, and protein per cell in the lag phase and the rate of cell-division showed no correlation. The DNA remained at a constant level, whereas the amounts of RNA and protein increased in both the lag phase and the log phase. A study of the same factors, at different time intervals in regenerating rat liver, by Hammarsten *et al.* (246) and by Anderson & Åqvist (247, 248, 249), and with regard to hemoglobin formation (250), points to a direct correlation between protein synthesis and RNA content and formation as viewed from coinciding maxima of time curves. Yet, as the authors point out, even if nucleic acids may have a primary role in an assumed interrelation with protein synthesis, it is hard to draw any conclusion if a rise in total RNA is a prerequisite for an increase in protein synthesis. A recent paper of Pardee (251) on the formation of adaptive enzymes in mutants of *E. coli* gives some interesting views on this question. According to Pardee only a small fraction of RNA actually partakes in protein synthesis, the bulk of RNA being without influence. Such findings might explain the correlation of RNA formation with protein synthesis despite the relative independence of amounts produced, in the case of selective inhibition of the synthesis of one or the other. The observation, for example, of Hokin & Hokin (252) that in pancreas slices RNA failed to increase during carbamyl-choline stimulation of enzyme production, falls in this category.

In view of the frequent though guarded suggestion that nucleic acids serve as the template, or guide, in the polycondensation process which leads from amino acids to proteins, these views imply that sufficient amounts of RNA or DNA should be available before protein synthesis takes place. In other words, the synthesis of nucleic acids must in some way precede protein synthesis. In the turnover rates of RNA and protein there are some instances where the turnover rate of RNA reaches a maximum before that of protein, as in the investigation of Chantrenne & Koritz on the incorporation rate of glycine-1-C^{14} in RNA and protein of reticulocytes (253). In this connection a most interesting experiment of Gale & Folkes should be mentioned, since it provides some evidence that RNA under some conditions may actually stimulate protein synthesis (254). The system examined consisted of sonic-disrupted cells of *Staphylococcus aureus* in which an increase of total proteins could be observed after administration of RNA preparations. It remains to

be seen, however, whether these experiments with disrupted cells and isolated RNA preparations reflect the situation in intact cells. If RNA and DNA actually participate as small fractions of activated compounds the scheme of Lipmann (188) is again called to mind. According to this nucleic acids could function as the high-molecular structure, be pyrophosphorylated by ATP, temporarily fix amino acids at certain discrete distances from each other, prior to polycondensation. This idea, however, stresses the importance of nucleic acids for protein synthesis, notwithstanding the importance of protein catalysts for polynucleotide formation. Both processes may be independent of each other; they may be closely related; or they may even be two aspects of the same process.

The role of particulate cellular constituents in protein synthesis.—A recent monograph on the chemistry and physiology of mitochondria and microsomes by Lindberg & Ernster (255) with about 300 references to this difficult subject has appeared. The chapter on protein synthesis with special respect to the particulate components of the cell is well worth reading. On the basis of an extensive review of the literature and from their own experiments the authors hold the view that the microsomes might be the structures in which the protein-synthesizing activity of the nucleus is brought to completion. The ribonucleoproteins, derived from the nucleolus, are probably the active principles for the protein metabolism of the microsomes. The role of the interaction, or exchange of material, between mitochondria and microsomes, as demonstrated by the pioneer work of Siekevitz (256), has been further studied by Hultin (257) in connection with an investigation of the incorporation rate of alanine-N^{15} in protein fractions of the sea-urchin embryo. That the intactness of RNA in microsomes appears to be indispensable for protein synthesis *in vivo* has recently been observed by Oota & Osawa (258) in a study of cell particulates in homogenates from growing beans. The authors find that the release of RNA from microsomes coincides with a complete inhibition of protein synthesis. This view is strongly supported by a work ofAllfrey, Daly & Mirsky (259). Following the procedure of Siekevitz (256) they prepared a suspension of rat liver mitochondria to which was added a microsome preparation from mouse pancreas and DL-alanine-1-C^{14}. In a parallel experiment the microsome material was preincubated with crystalline ribonuclease for 15 minutes at 37°. Comparing the specific activities of the microsome protein after 30 minutes contact with labelled alanine it was found that the ribonuclease-treated microsome protein had only 46 per cent of the radioactivity observed in the control preparation. Thus many indications point to microsomes as the actual *loci* for synthesis of cytoplasmic protein.

With regard to protein synthesis in cell nuclei and the possible participation of DNA, some experiments of Allfrey (260) are of special interest. Isolated nuclei of calf thymus tissue were shown to incorporate alanine-1-C^{14} at a certain rate, which was considerably increased by addition of an energy source, in this case α-ketoglutarate. Pretreatment of the nuclei with desoxyribonuclease, however, impaired the incorporation of alanine to a consider-

able extent, whereas ribonuclease had no effect. Judged from these experiments and recalling the restoration, by DNA, of amino acid incorporation in disrupted *Staphylococci* treated with desoxyribonuclease [Gale & Folkes (254)] it appears that DNA may play a role in the synthesis of nuclear proteins analogous to that of RNA with regard to cytoplasmic proteins. The possible influence of DNA on the latter process is, however, another question. Cohen & Barner (261) have found that in a thymine-requiring mutant of *Escherichia coli*, where DNA synthesis is low, or absent, the cytoplasmic synthesis of xylose-isomerase could still be induced by xylose. Some recent experiments of Hammarsten & co-workers (262) definitely show that in the lag phase of *E. coli* the active participation of DNA, as regards rate of formation and incorporation of nitrogen, is considerably delayed, beginning to show up only at a time period, where RNA and cytoplasmic proteins have already reached their respective maxima of incorporation rates. It might turn out that protein synthesis in nuclei and in the cytoplasm ordinarily represent two separate series of events, that become interrelated only during the active phases of cell division.

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NUCLEIC ACIDS[1]

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INTRODUCTION

During the past three years considerable advances have been made in our knowledge of the structure of the nucleic acids and their degradation products [Todd (1)]. It is proposed to review this work, with emphasis on material which has come to hand during the year to November, 1954. Methods for the isolation of nucleic acids were considered in some detail in last year's Review so that this aspect will not be stressed. A large amount of fundamental work has been published dealing with the elucidation of the biosynthetic pathways leading to the nucleotides and nucleic acids. This will not be discussed here, since it warrants more detailed consideration than space permits.

NUCLEOSIDES AND RELATED COMPOUNDS

The structures of the nucleosides derived from ribonucleic acids have been completely elucidated in earlier work. The final problem in the structures of the deoxyribonucleosides resides in the configuration at the glycosidic centre. This has been shown to be β, in the case of deoxyadenosine and deoxycytidine [Anderson, *et al.* (2)]. The demonstration depended on the formation of *cyclo*deoxynucleosides from the 5′-*O*-tosylnucleosides analogous to the *cyclo*-compounds formed in the ribonucleoside series [Clark, Zussman & Todd (3)]. It is probable that thymidine and deoxyguanosine are likewise, 2-deoxy-β-D-ribofuranosides. This is of some importance since structural proposals for the macromolecular deoxyribonucleic acids, which are discussed later, depend for their validity on this feature.

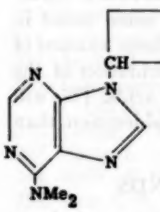
Details of the identification in and isolation from bacteriophage DNA of 5-hydroxymethylcytosine by Wyatt & Cohen (4) and of its deoxyriboside [Cohen (5)] have been given. The base appears to replace cytosine completely in the DNA[1] of T_{even} bacteriophages.

Although not related at present to the nucleic acids, several naturally occurring nucleosides deserve mention here. Bendich & Clements (6) have revised the formulation of vicine and its aglucone divicine. They give good evidence, based on chemical and spectral comparisons with analogous compounds, that vicine is the 5-*O*-β-D-glucopyranosyl derivative of 2,4-diamino-5,6-dihydroxypyrimidine. Brown & Weliky (7) have synthesised 9-β-D-ribofuranosylpurine by two routes and have identified it with the toxic product nebularine from the mushroom *Agaricus* (*Clitocybe*) *nebularis* Batsch.

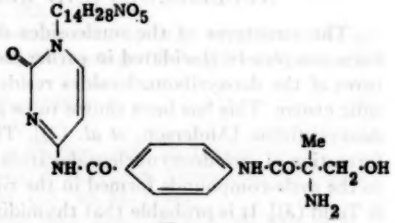
[1] The following abbreviations are used in this chapter: DNA for deoxyribonucleic acid; RNA for ribonucleic acid; TMV for tobacco mosaic virus; TYMV for turnip yellow mosaic virus.

[Löfgren & Lüning (8)]. Several syntheses in the glycosylbenziminazole series potentially applicable to nucleoside preparation have been recorded. These include the reaction of acetohalogen sugars with excess of 4,5-dimethylbenziminazole in which both α- and β-anomers are obtained [Johnson *et al.* (9)] and ring closure with a variety of reagents of 2-amino-4,5-dimethyl-N-glycosylaniline [Heyl *et al.* (10)].

Degradative studies on the mould product puromycin from *Streptomyces alboniger* have led to its formulation as 6-dimethylamino-9-(3′-*p*-methoxy-L-phenylalanylamino-3′-deoxy-β-D-ribofuranosyl)-purine (I;R = methoxyphenylalanyl residue) [Waller *et al.* (11); Hutchings *et al.* (12)].



I



II

Removal of the amino acid side chain affords the "aminonucleoside" (I;R = H). A 5′-mesyl derivative undergoes ready cyclisation to the 5′,N^3-*cyclo*nucleoside indicating the β-linkage at the glycosidic centre [Baker, Joseph & Williams (13, 14)]. The syntheses of 6-dimethylaminopurine [Elion *et al.* (15); Baker *et al.* (16)] and of 3-amino-3-deoxyribose and derivatives thereof [Baker & Schaub (17, 18)] have been followed by the total synthesis of puromycin itself [Baker *et al.* (19)]. The novel nucleoside synthesis involving a condensation of the chlorotitanium complex of an unstable substituted 1-chlorofuranose sugar with a chloromercuripurine may be expected to have wider application. The antitubercular antibiotic, amicetin, isolated from a strain of *Streptomyces* by Hinman, Caron & DeBoer (20) has been shown to be a derivative of cytosine to which the partial structure (II) has been given [Flynn *et al.* (21)].

Fox, Chang & Davoll (22) have described, in abstract, a new route for pyrimidine nucleoside synthesis which may be more versatile than the earlier Hilbert-Johnson method. Tetraacetyl glucopyranosyl-thymine and -thiour-

acil are obtained in good yield by condensation of acetobromoglucose with the mercurichloride of the corresponding pyrimidine.

Two members of the Vitamin B_{12} group have been isolated from calf and pig manure and shown to contain 2-methyladenine and 2-methylhypoxanthine in place of the dimethylbenziminazole present in Vitamin B_{12} itself [Brown & Smith (23); Dion *et al.*, (24)]. It is interesting to recall that Bergmann & Feeney (25) isolated a nucleoside from sponges, in addition to spongothymidine, which gave an amino-hydroxy-methylpurine on hydrolysis.

The mechanism of oxidative degradation of uric acid has been reinvestigated by Brandenberger (26) and by Dalgliesh & Neuberger (27) in view of its importance in tracer studies of purine biosynthesis. The reduction of some purines and pyrimidines by zinc in acid solution and in the polarograph has been followed spectroscopically by Hamer, Waldron & Woodhouse (28). Burke (29) found that the reduction of pyrimidine nucleosides by sodium and alcohol in liquid ammonia, followed by removal of sodium ions by a cation-exchanger, permits the direct isolation of the sugar component. The method appears to have advantages over that involving catalytic hydrogenation, and using it he was able to show the presence of 2-deoxy-D-ribose in deoxycytidine and thymidine.

Ultraviolet spectroscopic studies have been made of xanthine and derivatives [Cavalieri *et al.* (30)] and of other natural and synthetic purines [Mason (31); Albert & Brown (32)]. The complexes formed between purines and aromatic compounds are considered on spectroscopic grounds to be held together by slight forces which owe their origin to mutual polarisation [Booth, Boyland & Orr (33)]. Bredereck *et al.*, (34, 35) describe further syntheses in the xanthine series. Preparations for [2-^{14}C]- and [8-^{13}C]-adenine are given [Paterson & Zbarsky (36); Gordon (37)]. A careful x-ray crystallographic study of uracil has been made [Parry (38)].

The chemistry of ribose has received some attention. Separations of methylated riboses by paper chromatography and ionophoresis in presence of borate have been described [Barker & Smith (39); Brown, Magrath & Todd (40)]. Barker & Smith (41) have resolved mixtures of the anomeric methyl ribo-furanosides and -pyranosides on cellulose columns. Bredereck & Gremer (42) have prepared mono-, di-, and tri-trityl ethers of ribose. Fletcher and co-workers have continued their interesting studies of the benzoates of ribose. Derivatives of 2,3,5-tri-*O*-benzoylribose (43) and 3,5-di-*O*-benzoylribose (44) are described. These furanose derivatives, because of their greater stability than the acetates, should have useful synthetic application in the nucleoside series.

Synthesis in the deoxyribonucleoside series has been precluded heretofore, mainly by lack of 2-deoxy-D-ribose in sufficient quantity, earlier syntheses of this sugar being of little preparative value. Four syntheses have been recorded recently (45 to 49). Richards (47, 48) obtains 2-deoxy-D-ribose in 31 per cent yield by a Ruff degradation of glucometasaccharinic

acid, which is in turn formed in high yield from 3-*O*-methylglucose by the action of dilute alkali. Sowden (49) similarly utilises the crude saccharinic acids obtained from glucose by treatment with concentrated sodium hydroxide. Kenner & Richards (50) describe syntheses from glucose of 5-*O*-benzyl- and 3,5-*O*-benzylidene-2-deoxyribose, compounds potentially amenable to conversion into deoxyribofuranosides. Laland & Overend (51) describe the isolation of deoxyribose from deoxyribonucleic acids using enzymic and ion-exchange methods.

Ribonucleic Acids

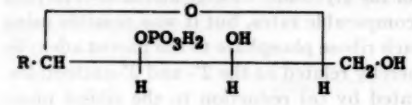
During recent years many aspects of the chemical structure of the ribonucleic acids have become clear, and it would seem timely to discuss the present position. Brown & Todd (52) on the basis of their mechanism of hydrolysis of ribonucleic acids were able to propose structures for these acids which accounted for the isolation of nucleoside-2′ and -3′ phosphates, the *a* and *b* nucleotides of Carter (53) and Cohn (54) from chemical hydrolysates, and of nucleoside-5′ phosphates [cf. Cohn & Volkin (55, 56)] from enzymatic degradations. The 3′,5′- or 2′,5′-linked polynucleotide structures were favoured, and it was further pointed out that branches could be accommodated, if required, although specified limitations had to be imposed in view of the requirement that alkaline hydrolysis should yield only mononucleotides. Two possible types of branching were envisaged involving side chains to the main (3′,5′-linked) "backbone": (*a*) arising from $C_{2'}$ of the branch-point nucleoside and (*b*) by incorporating phosphotriester linkages as postulated earlier by Gulland and co-workers (57). Based on these general formulations, solutions to essentially four problems were required before complete definition of any ribonucleic acid structure could be given. The problems were: (*a*) the orientation of the phosphoryl group in the isomeric *a* and *b* nucleotides, (b) a decision between 2′,5′- and 3′,5′- as the main internucleotidic linkage points, (*c*) the nature and extent of any branching, if present, and (*d*) the nucleotide sequence in ribonucleic acid chains. Considerable progress has been made and recent evidence which has accrued will be summarised.

Mononucleotides.—Synthesis of adenylic acids *a* and *b* by phosphorylation of 5′-*O*-trityladenosine with dibenzylphosphorochloridate showed that these acids must be adenosine-2′ and -3′ phosphate, but not necessarily respectively [Brown & Todd (58)]. Barker (59) has recently synthesized them by the action of phosphoryl chloride in wet pyridine on ditrityladenosine, a process leading readily to the radioactive P^{32} compounds. Recent work has defined completely the orientation of the phosphoryl group in the isomeric acids. Cavalieri (60) has measured their pK values and densities of their solutions. The greater density and lower pK value of the amino group in the *b* isomer was taken to indicate greater charge separation in the zwitterion, and hence it was concluded that the *a* and *b* isomers were the

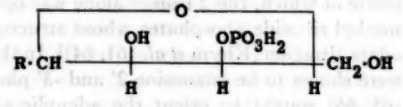
2′- and 3′-nucleotides respectively. The same conclusion had been reached by Khym *et al.* (61), of whose work further details have now been given [Khym & Cohn (62)]. Acid hydrolysis of each of the isomeric adenylic acids by the acid form of the sulphonic acid resin, Dowex-50, leads to mixtures of ribose phosphates, predominately the 2- and 3-isomers. These can be estimated after separation by ion-exchange chromatography in borate solutions [cf. Khym & Cohn (63)]. Fission of the glycosidic linkage and acid-catalysed phosphate migration proceed at comparable rates, but it was possible using short hydrolysis times, to relate each ribose phosphate to the parent adenylic acid. The *a* and *b* isomers were thereby related as the 2′- and 3′-nucleotides. The ribose phosphates were oriented by (*a*) reduction to the ribitol phosphate of which, the 2-isomer alone was optically active and (*b*) formation of methyl riboside phosphates whose structures could be determined by periodate titration [Khym *et al.* (61, 64)]. In the same way guanylic acids *a* and *b* were shown to be guanosine-2′ and -3′ phosphate respectively. Brown, *et al.* (65, 66) sought to orient the adenylic acids unambiguously by synthetic means which precluded phosphoryl migration. Acetylation of 5′-*O*-acetyladenosine gave a crystalline x:5′-di-*O*-acetyladenosine which on phosphorylation and removal of protecting groups yielded adenylic acid *a* uncontaminated by any of the *b* isomer. Monotosylation of the original diacetyladenosine and orientation of the tosyl group by methylation proved that it occupied the 2′-position. This demonstrated conclusively that adenylic acid *a* is adenosine-2′ phosphate. Concurrent x-ray crystallographic studies by Woolfson and Cochran [Brown *et al.* (65)] showed that adenylic acid *b* is adenosine-3′ phosphate.

Turning to the pyrimidine nucleotides, Cavalieri (60) concludes from physical measurements (see above) that cytidylic acids *a* and *b* are cytidine-2′ and -3′ phosphate. Michelson & Todd (67) reach the same conclusion from a comparison of the infrared spectra and other properties of the cytidine and deoxycytidine phosphates. Baron & Brown (68) degrade these acids to ribose phosphates by hydrazine, conditions under which phosphoryl migration cannot take place. They also reach the same conclusions as the above authors. Earlier work has related the uridylic acids to the cytidylic acid isomers so that it seems firmly established that in both the purine and the pyrimidine nucleotides, the *a* and *b* isomers are, respectively, the nucleoside-2′ and -3′ phosphates (III and IV; R = purine or pyrimidine residue). The earlier indication that all the *b* isomers (and hence all the *a* isomers) belonged to the same series depending on enzymatic dephosphorylation by the specific *b*-nucleotidase, is thus confirmed. [Shuster & Kaplan (69)]. Brown (70) has suggested that the nucleoside-2′ and -3′ phosphates and their esters are eluted from ion-exchange columns in that order (the *a* and *b* nucleotides were originally defined on these grounds) because of the small differences in pK values of their dissociating groups, or probably more precisely, as a result of those factors which give rise to the pK value differences [Cavalieri (60)].

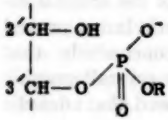
Ribonucleotide esters.—The hydrolysis of ribonucleic acids was shown to proceed in the same way as that of simple esters of the nucleoside-2′ and -3′ phosphates. [Brown & Todd (52)]. It was suggested that an ester (V), in acid or alkaline solution, yielded the cyclic phosphate (VI) which then formed the isomeric nucleotides (e.g., VII) by random fission of the cyclic O-P bonds. Formation of VI was considered to proceed via an intermediate



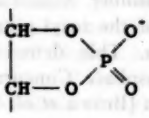
III



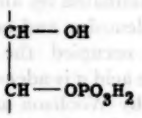
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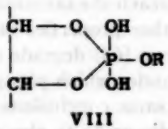
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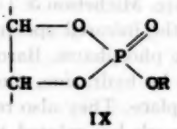
VI



VII



VIII



IX

(VIII or IX). More recently these authors (71) have discussed the hydrolytic mechanism and have pointed out that instead of involving the intermediate (IX) which was invoked for simplicity in representation, the process should be considered as an attack by the vicinal hydroxyl group on the P=O band, catalysed by hydroxyl or hydrogen ions, with simultaneous removal of R as an alkoxy anion, possibly with the intervention of a quinquecovalent complex (VIII or an ionised form). This view of the mechanism finds confirmation in the experiments of Lipkin, Talbert & Cohn (72) who showed that alkaline hydrolysis of yeast ribonucleic acid in H_2O^{18} yields mononucleotides containing approximately one atom of O^{18} per atom of phosphorus; two atoms of O^{18} would be expected to be incorporated if a true cyclic triester intermediate was involved. It is interesting to note that they

did not find exactly one O^{18} incorporated but, on the average 0.85 atoms O^{18}/P. They were unable to account for this, but, in the absence of any systematic error in estimation, a probable explanation can be given. O^{18} incorporation can only occur during fission of an internucleotidic linkage; the terminal monoesterified phosphate will be unaffected. If n is the number of nucleotides in the polynucleotide then $n\text{-}1/n = 0.85$ or $n = 6.6$. This result, if taken on its face value, shows that the nucleic acid used was of rather low molecular weight, corresponding to a hexa- or hepta-nucleotide. Low molecular weight in yeast nucleic as found by end-group determination was earlier indicated by Markham & Smith (73). It is suggested that the O^{18} incorporation method might find use in molecular weight determination and could, in theory, give evidence of the presence or absence of phospho-triester branching in ribonucleic acids.

A new method for the preparation of nucleoside-2′,3′ (cyclic) phosphates depends on the action of dicyclohexylcarbodiimide on the 2′- or 3′-nucleotide [Brown (74)]. The method has been developed by Dekker & Khorana (75) who show that with adenylic acid the 2′,3′-phosphate is first formed and that this is converted by excess of the reagent in aqueous pyridine to two isomeric substances of the type (X). These are hydrolysed

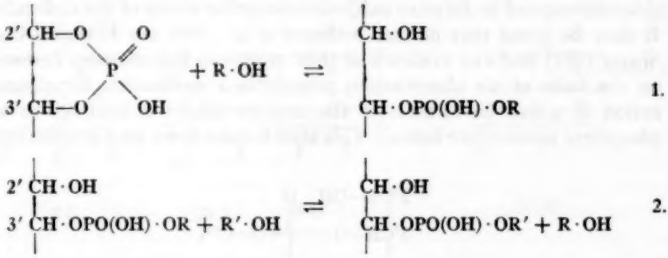
$$\begin{array}{l} \text{—CH(OH)·CH—OPO(OH)·N}(C_6H_{11})\text{·CO·NH·}C_6H_{11} \\ \quad\quad\quad\

cytidine-3′ phosphate are hydrolysed by this enzyme; the 2′-isomers are unaffected [Brown & Todd (71)]. This gives evidence as regards linkage of the pyrimidine nucleotides in the nucleic acids. In regard to the purine nucleotide linkage Brown, Heppel & Hilmoe (78) found that cytidine-3′ and adenosine-3′ benzyl phosphate were hydrolysed to the nucleotide by a spleen fraction [Hilmoe & Heppel (79)]. Fractions from intestinal mucosa, rye grass, and potato also effected the hydrolysis; in all cases the 2′-esters were unaffected. The spleen fraction hydrolyses ribonucleic acids and certain ribonuclease limit-polynucleotides to the 3′-mononucleotides and, since the hydrolysis does not appear to involve a cyclic intermediate it was tentatively concluded that the 3′-position is involved in the internucleotidic linkage [Heppel, Markham & Hilmoe, (80); Volkin & Cohn (81)]. This was rendered more likely in view of the finding that nucleoside-2′,3′ phosphates are hydrolysed by the same preparation to nucleoside-2′ phosphates [Heppel *et al.* (82)]. A degradation method, the mechanism of which is discussed below, which does not involve phosphoryl migration has been applied to a variety of dinucleotides and trinucleotides from ribonuclease digests; in each case purine nucleoside-3′ phosphates are obtained [Whitfeld & Markham (83); Whitfeld (84)]. The present evidence, therefore, establishes that the ribonucleic acid internucleotidic linkage involves the 3′(*b*)- and 5′-hydroxyl groups of the nucleoside residues.

Nucleases.—Volkin & Cohn (85) have made a comprehensive study of the products of pancreatic ribonuclease digestion. They show that ion-exchange chromatography on a 2 per cent cross-linked Dowex-1 resin column allows the separation of many oligonucleotides. Twenty-three such substances ranging from di- to tetranucleotides have been structurally characterised by a combination of chemical and enzymatic (snake venom diesterase, bone phosphomonoesterase, and barley 3′-nucleotidase) degradation. Other less well characterised substances have also been obtained. Ribonuclease action is discussed in the light of the structures and proportions of the respective oligonucleotides isolated, and earlier views on the specificity of the enzyme have been confirmed.

As mentioned above, Brown & Todd (71) showed that ribonuclease hydrolyses simple esters of pyrimidine nucleoside-3′ phosphates through the intermediate cyclic phosphate to nucleoside-3′ phosphates. Heppel & Whitfeld (86) have made the interesting observation that the first stage in this hydrolysis is reversible. Cytidine-2′,3′ phosphate with methanol, in presence of ribonuclease, forms cytidine-3′ methyl phosphate (see Equation 1). Uridine cyclic phosphate also participates in the synthetic reaction, but purine derivatives are inactive, in consonance with the known specificity of the enzyme. Further they showed that an exchange reaction (see Equation 2) occurs, catalysed by ribonuclease, e.g., with cytidine-3′ benzyl phosphate and methanol; cytidine-3′ methyl phosphate and benzyl alcohol are produced, the reaction competing favourably with hydrolysis to cytidine-3′ phosphate.

With the above-mentioned spleen enzyme, exchange reactions are observed. In accord with the wider specificity of this enzyme preparation, esters of adenosine-3′ phosphate act as substrates. A nucleoside may act as the alcohol component of the ribonuclease-catalysed system [Heppel, Whitfeld & Markham (87)]. Thus cytidine with uridine-2′,3′ phosphate yields uridylylcytidine (uridine-3′ cytidine-5′ phosphate) (Equation 1). Dinucleotides may act as donor molecules with the spleen enzyme; for example, adenylyluridylic acid and cytidine afford adenylylcytidine and uridylic acid. Products of greater complexity, such as trinucleoside diphosphates, are also formed, but in smaller yield. In each case a 3′,5′-linkage appears to be formed as in the natural polynucleotides. These results may invalidate certain structural conclusions which might be drawn from nucleotide sequences in polynucleotides

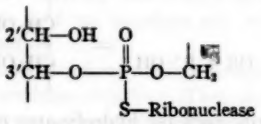


derived from partial ribonuclease hydrolysates of ribonucleic acids. Thus, the cyclic triuridylic acid isolated by Markham & Smith (88), which suggested the presence of that sequence in the intact RNA, may have derived, in part, from the competing synthetic action of the enzyme. This proviso does not, however, apply in the case of complete hydrolysis.

In parenthesis, the question may be asked as to whether these transesterification reactions are of importance for nucleic acid synthesis and turnover in the living cell [cf. Hokin & Hokin (229)]. It may be pertinent to the question that the growth requirement of certain uracil-requiring microorganisms is satisfied by some dinucleotides and by uridine-2′,3′ phosphate [Merrifield & Wooley (89); Rose & Carter (90)]. There is some indication that RNA synthesis is apparently increased by ribonuclease when injected into amphibian eggs [Ledoux *et al.* (91)] or when applied to living onion root-tip cells [Brachet (92)]. Roth (93) on the other hand cites experiments which suggest that one of the intracellular functions of ribonucleases is to degrade ribonucleic acid, which acts as a template for protein synthesis, thus liberating the newly formed protein chain. The idea that polyphosphates of the adenosinetriphosphate type are precursors of nucleic acids is more in line with current biochemical thought. In addition to the adenine nucleotides, it is now known that the mono-, di-, and triphosphates of guanosine, cytidine, uridine, and inosine are present in tissues (94 to 98), and the ex-

periments of Potter and co-workers (99, 100) show with some certainty that these are involved as ribonucleic acid precursors.

Ledoux in a series of papers discusses the active groups in ribonuclease. He notes the inhibitory action of sodium chloromercuribenzoate, reversible by reduced glutathione and relates this to thiol group blocking. An initial activation by this reagent is associated with interaction at the polarisable C=O group of the pyrimidine nucleotide residues in the RNA undergoing hydrolysis (101, 102). Dickman *et al.* (103), however, are unable to observe this activation. Evidence is given that reducing agents activate and oxidising agents inactivate the enzyme, reversibly, and that in redox-buffer solutions the activity is a function of the redox potential (104). A chromatographic study of ribonuclease on Amberlite IRC-50 indicates that the various fractions correspond to different oxidation-reduction states of the molecule (105). It may be noted that neither Anfinsen *et al.* (106) nor Fraenkel-Conrat & Singer (107) find any evidence of thiol groups in this enzyme. Ledoux (108) on the basis of his observations postulates a mechanism for ribonuclease action in which he formulates the enzyme-substrate complex as a thiol-phosphate triester (see below). This then breaks down by a process involving



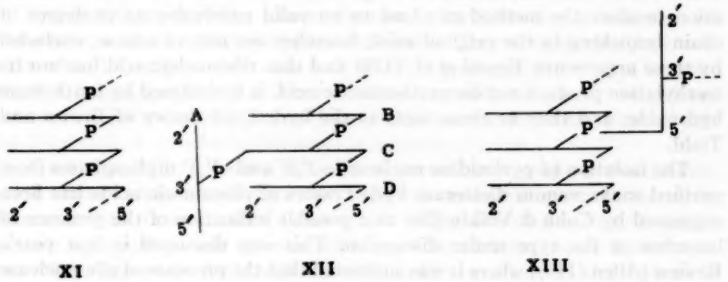
the hydroxyl group at $C_{3'}$. Long (230) has reviewed present views of ribonuclease action.

Durand & Thomas (109) find that ribonuclease causes the degradation of thymic (apurinic) acid. McDonald & Kaufmann (110) confirm this and show that two other basic proteins, lysozyme and cytochrome-*c*, are effective at pH 6.5. The evidence suggests that none of the proteins is acting enzymically. It may be that they are promoting a base-catalysed fission by a mechanism analogous to that discussed by Cramer (231) involving inclusion compounds. Further x-ray crystallographic work on the enzyme is described [Carlisle, Scouloudi & Spier (111); Wrinch (112); Low (113)]. Considerable progress is being made toward establishing the amino acid sequence in this protein [Anfinsen *et al.* (106); Hirs *et al.* (232)].

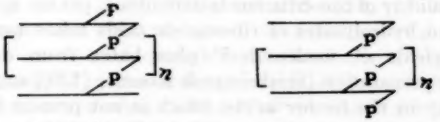
It is becoming clear that nucleases other than pancreatic ribonuclease exist. Roth (114) lists some animal and bacterial sources of nuclease activity. He shows that two ribonucleases are present in rat liver and kidney (pH optima 5.8 and 7.8) and studies their distribution in various cell fractions [cf. also de Lamirande *et al.* (115)]. Maver & Greco (116) have achieved considerable purification of a ribonuclease from calf spleen by an anion-exchange column method. It seems likely that specificities differing from that of pancreatic ribonuclease will be encountered in the nucleolytic enzymes isolated from diverse sources. Brown, Heppel & Hilmoe (78) point out that the simple

nucleotide esters of known structure provide valuable substrates for specificity studies and for following the fractionation of these enzymatic activities.

Chain-branching and nucleotide sequence.—The studies discussed above indicate with some certainty that the ribonucleic acids are 3′,5′-linked polynucleotides. XI represents a fragment of such a chain (P is the phosphodiester bridge and the lines indicate the nucleoside residues). Brown & Todd (52) suggested that, if branching occurred, it could do so in two ways which would still permit complete hydrolysis to mononucleotides. These are (*a*) branching on sugar as in XII such that the phosphodiester grouping joins $C_{2'}$ of the



XI XII XIII



XIV XV

branch-point nucleoside to $C_{3'}$ of the first nucleoside residue in the branch and (*b*) branching on phosphorus, i.e., by the introduction of phosphotriester groupings (XIII) as suggested by Fletcher, Gulland & Jordan (57).

Branching on the sugar was proposed independently by Anderson *et al.* (117) as a result of studies involving methylation of yeast nucleic acid. Methylation with methyl iodide and silver oxide followed by hydrogenation and hydrolysis yielded a mixture of ribose, monomethylribose, and dimethylribose. On the grounds that full methylation had been achieved and that the nucleic acid was not degraded during methylation, they considered that the ribose had originated from branch points [(XII); residue C], the dimethylribose from branch termini (A) and the monomethylribose from the remaining nucleoside residues (B, D) in the chain. The proportion of ribose to monomethylribose indicated a high degree of ramification. These conclusions have been criticized by Brown, Magrath & Todd (40). In a study of the methylation of uridylic acid *b* (uridine-3′ phosphate), they found (*a*) that

methylation is slow so that many treatments are necessary for completion, (*b*) that phosphoryl migration must occur during the process, since both 2,5- and 3,5- di-*O*-methylribose can be isolated, and (*c*) that very unstable intermediates are formed during the methylation. They argued that since methylation of a nucleotide ester is analogous to methylation of a polynucleotide and since phosphoryl migration and hydrolytic fission are consequent on each other, fission of some of the internucleotidic linkages of ribonucleic acid during methylation should inevitably occur. This would increase the number of end groups giving rise to dimethylribose. In absence of much better evidence for the molecular weight of a ribonucleic acid and its methylation product the method can lead to no valid conclusion as to degree of chain branching in the original acid; branches are not, of course, excluded by these arguments. Egami *et al.* (118) find that ribonucleic acid but not its methylation product nor deoxyribonucleic acid, is hydrolysed by lanthanum hydroxide, and they fit these facts to the hydrolytic theory of Brown and Todd.

The isolation of pyrimidine nucleoside-2′,5′ and -3′,5′ diphosphates from purified snake venom diesterase hydrolysates of ribonucleic acids has been suggested by Cohn & Volkin (56) as a possible indication of the presence of branches of the type under discussion. This was discussed in last year's Review [Allen (119)] where it was suggested that the presence of ribonuclease in the enzyme preparation could account for the production of these substances. The validity of this criticism is undoubted, yet the appearance of the diphosphates in hydrolysates of ribonucleic acids taken together with the quantitative yields of nucleoside-5′ phosphates from deoxyribonucleic acids with this preparation [Sinsheimer & Koerner (120)] suggest some type of discontinuity in the former acids, which is not present in the straight-chain deoxyribonucleic acids. Brown, Fried & Todd (121) have indicated one way of discovering any such branches which depends on stepwise degradation of polynucleotides. Present evidence [cf. Volkin & Cohn (85)] indicates that the polynucleotides from ribonuclease digests are linear molecules. This being so the results discussed above imply that if branching on sugar residues occurs, the branchpoint (C in XII) and the first residue in any branch (A) must be a pyrimidine nucleoside.

No direct evidence for the presence of phosphotriester-linked branches in ribonucleic acids has been given. Postulation of this feature (e.g., partial formula XIII) led to a ready explanation of the larger number of end-groups carrying monoesterified phosphate, indicated by electrometric titration, than could be accounted for by a linear polynucleotide [Fletcher *et al.* (57); Jordan (122)]. Observations bearing on the point were made by Brown, Magrath & Todd (40), when they found that substances of the type RO.PO $(OMe)_2$ (R = nucleoside-2′ or -3′ residue) were unstable. For example, when chromatographed in the *iso*propanol-ammonia solvent (pH *ca.* 9) they were converted to the monomethylester and ran as such. Further experiments (123) have shown the extreme instability, even at neutrality, of this struc-

ture which is, of course, a simple analogue of the phosphotriester grouping proposed for ribonucleic acids. These findings indicate that very much better evidence will be required before the presence of this type of branching in any isolated RNA can be accepted. On this view, the results of titrimetric studies could be accomodated if it were assumed that the nucleic acids studied consisted of rather low molecular weight material [cf. Markham & Smith (73)] or that polynucleotides titrate abnormally. Evidence concerning the latter point might readily be obtained by electrometric titration of homogeneous oligonucleotides which are now available [*inter alia* Markham & Smith (88); Volkin & Cohn (85)].

Ribonucleic acids.—The above discussion leads to the formulation of ribonucleic acids as 3′,5′-linked polynucleotides. They may be straight-chain molecules (XI) or carry branches as in (XII). The degree and type of branching, if it occurs, is still a very controversial issue, and, indeed may vary from one nucleic acid to another. Nevertheless these general structures form a basis for discussion of the ribonucleic acids as macromolecules.

Markham, Matthews & Smith (124) have hydrolysed several nucleic acids with alkali and have separated the products by ionophoresis on paper. Turnip yellow mosaic virus RNA yields only mononucleotides. Tobacco mosaic virus and potato virus X RNA yield, in addition, small amounts of two other types of products which they identify as nucleosides and nucleoside diphosphates. The authors find a simple explanation of these observations if it is assumed that two types of ribonucleic acids exist, viz., those in which the terminal mono-esterified phosphate lies at $C_{3'}$ or $C_{2'}$ (XIV) exemplified by TYMV[1] nucleic acid and those terminated by a phosphate group at $C_{3'}$ (XV) exemplified by the latter two acids. XIV would break down on hydrolysis to give only nucleoside-2′ and -3′ phosphates while XV would yield in addition a nucleoside from one end and a nucleoside-2′,5′ and -3′,5′ diphosphate from the other end of the chain. They find that TMV[1] and Potato virus X nucleic acids consist substantially of chains of type XV with some of type XIV. On the grounds that small amounts of purine nucleoside-2′,3′ phosphates can be demonstrated in ribonuclease digests of TYMV nucleic acid, Markham & Smith (88) postulated that certain nucleic acids may terminate in cyclic phosphate groups. It should be made clear, however, that any degradation of a type XV molecule, attendant on isolation, will yield molecules carrying a 2′-, 3′-, or 2′,3′-phosphate group in the terminal position. Thus it may be that all ribonucleic acids will be found to be of type XV and that the appearance of type XIV chains is a reflection of their degraded state.

The ratio of nucleoside diphosphate to mononucleotide is about 1:50 in hydrolysates of TMV nucleic acid. This would indicate a molecular weight similar to that found by end-group determination for the TYMV acid [Markham & Smith (88)] and considerably smaller than had been deduced earlier from physical measurements [Cohen & Stanley (125)]. However, Northrop & Sinsheimer (126) have studied the molecular weight of RNA

obtained from TMV by heat denaturation using the technique of light scattering. They obtain a value of $2.0-2.2\times10^6$ corresponding to all the nucleic acid (5.5 per cent) in the virus rod (estimated M, 40×10^6). They note instability in the nucleic acid which they suggest is perhaps attributable to enzyme action but may result from dissociation of weakly bound subunits. Watson (127) concludes, from an x-ray crystallographic study of TMV, that the virus rod consists of a helical arrangment of equivalent subunits round the longitudinal axis. He reaches a value for the subunit M of 35,000 which gives the number of subunits in the infective particle as about 1,200 [compare, Fraenkel-Conrat & Singer (107)]. Schmidt, Kaesberg & Beeman (128) have studied the small angle x-ray scattering from TYMV and consider that the virus particles are spherical (radius 140Å) with the nucleic acid inside the protein shell, in agreement with earlier work. The physical chemistry of viruses and virus nucleic acids has been reviewed [Markham (129, 130); Markham & Smith (131)].

Since Watson & Crick (132) proposed their theory of the macromolecular structure of DNA, discussed below, in which specific hydrogen bond pairing of bases (adenine—thymine and guanine—cytosine) occurs between two helically arranged polynucleotide chains, it has been of considerable interest to speculate whether the ribonucleic acids are like constituted. A clear cut answer to this question may not be immediately forthcoming for it seems likely that ribonucleic acids, as isolated, are in a more or less degraded condition. Moreover, if these acids are branched, physical measurements may be rendered more difficult in consequence of loss of symmetry. Rich & Watson (133) have taken x-ray photographs of a variety of RNA fibres which, however, do not have the high degree of orientation shown by DNA. The same spacings are noted in each, but differences from the x-ray diagrams given by deoxyribonucleic acids are evident. The evidence is taken to indicate that ribonucleic acids are long asymmetric molecules. In a discussion of some of the relations between RNA and DNA, bearing on the question as to whether specific base-pairing occurs in the former acids, they (134) have collected the published data on base composition of various ribonucleic acids. In most cases a purine/pyrimidine ratio of one is found, but in the case of the plant virus ribonucleic acids large deviations from unity are apparent. This is also clear from recent analyses by Dorner & Knight (135). Elson & Chargaff (136) have looked for regularities in RNA composition. In order to avoid errors as a result of degradation during isolation, they have made analyses on whole cells. Ratios of 1:1 are found for adenine/uracil, guanine/cytosine, and purine/pyrimidine. Systematic deviations are found in nuclear and nucleolar ribonucleic acids where, however, the ratio (guanine+uracil)/(adenine+cytosine) is 1:1. Thus the number of 6-keto groups equals that of 6-amino groups on the base residues, and on these grounds they consider the added possibility of purine-purine and pyrimidine-pyrimidine pairing in these nucleic acids. Implicit in these papers is the suggestion that macromolecular RNA, like DNA, is in the form of a double helix. Some further evidence addi-

tional to base composition and preferably of a crystallographic nature is necessary before such conclusions can be drawn with any certainty. Fraenkel-Conrat (137) finds that when RNA and the viruses containing this acid are treated with 1 to 2 per cent formaldehyde solution an increase in optical density and shift of λ_{max} to higher wavelengths occurs. The reaction appears to be associated with the base amino groups. It suggests that hydrogen bonds between the bases are very weak since DNA does not show these effects. Against this the increases in optical density of RNA solutions when subjected to degradative conditions [Mallette & Lamanna (138)] may find a common explanation with similar observations on DNA in terms of hydrogen bond rupture (see below).

Further studies on the incorporation of unnatural purines and pyrimidines into ribonucleic acids are described. Jeener & Rosseels (139) find evidence of incorporation of thiouracil into TMV, since hydrolysis of the isolated nucleic acid gives a compound having the characteristics expected of 2-thiouridylic acid. Matthews (140, 141) is unable to confirm this but gives evidence that 8-azaguanine is incorporated by the demonstration that alkaline hydrolysis of the virus nucleic acid affords material with the expected properties of 8-azaguanylic acid (*a* and *b* isomers). Mandel, Carlo & Smith (142) and Lasnitzki, Matthews & Smith (143) extend these observations to several other organisms and tissues. The latter authors are unable to detect incorporation into DNA. They suggest that because the pK value of the 6-hydroxy group of azaguanine is 2 to 3 units below that of guanine, incorporation of this base would affect adversely the hydrogen bonding system in the DNA double helix. This would imply, of course, that RNA and DNA are not analogously constituted in their macromolecular structures.

Ronwin (144) proposes structures for the nucleic acids which are radically different from those discussed above. He conceives them as having a polyphosphate or polymetaphosphate backbone esterified with nucleoside residues. Other unlikely features include pentacovalent phosphorus groupings of a type for which no true analogy exists, except possibly in transition states associated with phosphate ester hydrolysis. The compounds of the type $P(OAr)_5$ (Ar = aromatic residues), quoted by Ronwin, are rapidly decomposed by water and are from this and other points of view irrelevant to the discussion. Until evidence can be adduced which is incompatible with the present concepts of nucleic acid structure, Ronwin's formulation should not be seriously considered.

Deoxyribonucleic Acids

The discussion of deoxyribonucleic acid structure can be conveniently divided into two categories. The first relates to the general chemistry of these acids and the second to their macromolecular structure or conformation. In both these aspects very considerable progress has been made in recent years, and structural hypotheses have been put forward suitable for testing experimentally.

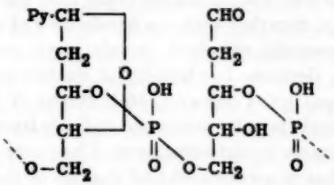
With regard to general structures the accumulated evidence has led to the view that these acids are linear polynucleotides in which 2′-deoxyribonucleoside residues are linked at the 3′- and 5′- positions by phosphodiester bonds. Their chemistry is in accord with this formulation [Brown & Todd (52); Dekker, Michelson & Todd (145)]. As regards the macromolecule, the proposal of Watson & Crick (132) that the structure should be considered as a double helix composed of two polynucleotide chains held together by hydrogen bonding between the base residues has provided a basis for discussion of many physicochemical aspects of DNA.

Degradative and synthetic studies.—Michelson & Todd (67) have continued their syntheses of the monodeoxyribonucleotides. Phosphorylation of 5′-*O*-trityldeoxycytidine and 3′,N^6-diacetyldeoxycytidine with dibenzylphosphorochloridate and then removal of protecting groups, yields the crystalline deoxycytidine-3′ and -5′ phosphates respectively. Very recently the 3′- and 5′- phosphates of deoxyadenosine and deoxyguanosine have been synthesized [Hayes, Michelson & Todd (146)]. These syntheses together with those of the isomeric thymidine phosphates [Michelson & Todd (147)] complete the preparations of the monophosphates of the more common naturally-occurring deoxyribonucleosides. In these papers the 5′-phosphates of the nucleosides are shown to be identical with the corresponding mononucleotides isolated quantitatively from enzymic digests of DNA [Hurst *et al.* (148); Sinsheimer & Koerner (120)]. This confirms the previously allotted structures based on studies using the enzyme 5′-nucleotidase, the specificity of which rested on activity against 5′-ribonucleotides [Carter (149)].

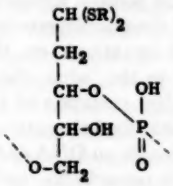
Sinsheimer (150) has hydrolysed the DNA of T_{2r}^+ bacteriophage with deoxyribonuclease followed by purified snake venom diesterase. Of the original DNA phosphorus, 62 per cent is liberated as mononucleotides, the rest being present as di-, tri-, and polynucleotides; this is in contrast to the almost quantitative production of mononucleotides from a variety of other deoxyribonucleic acids, mentioned above. Fractionation of the mononucleotides yields two new acids (H1 and H2) both of which are 5′-phosphates. H1 gives H2 on acid hydrolysis with liberation of glucose. Other hydrolytic conditions yield 5-hydroxymethyl cytosine and a glucosyl hydroxymethylcytosine. Thus the evidence points to the presence in this bacteriophage DNA of 5-hydroxymethylcytosine nucleotide residues carrying a glucose residue probably in glycosidic combination with the 5-hydroxymethyl group. This conclusion is reached independently by Volkin (233) from the results of enzymatic and acidic hydrolyses. He finds, further, that glucose is associated mole for mole with hydroxymethylcytosine and is also present in T_4 and T_6 (but not T_1) phages. Mayers & Spizizen (151) in describing the analysis of bacteriophage DNA isolated by an improved method, note a sugar to phosphorus ratio greater than unity. This is probably to be accounted for by Sinsheimer's and Volkin's observations. Weed & Courtenay (152) have isolated 5-hydroxymethyldeoxycytidine diphosphate from an

acid hydrolysate of bacteriophage DNA. They also find a polynucleotide in sperm DNA hydrolysates containing thymine and cytosine with a phosphorus/base ratio of two. It is not at all obvious how this molecule should be formulated since it corresponds, e.g., to a dinucleoside tetraphosphate, and further clarification would be welcome. Dekker, Michelson & Todd (145) point out the likelihood that the pyrimidine deoxynucleoside-3′,5′ diphosphates produced on acid hydrolysis of DNA arise from positions in the chain where one pyrimidine is flanked by purine nucleotide residues. It would follow that a dinucleoside triphosphate might be expected to arise when two pyrimidine nucleotides in sequence were bounded by purine nucleotide residues.

These acid degradation products must result from the hydrolysis of initially produced apurinic acids (XVI), which themselves are formed under very mild acidic conditions [Tamm *et al.* (153)]. Laland (154) prepares these



XVI



XVII

substances by setting aside solutions of deoxyribonucleic acids with an acidic ion-exchange resin. Since quantitative removal of purine bases is effected, he utilises this for analytical purposes. The apurinic acids, by virtue of the free deoxyribose residues present in the molecule, should be susceptible to very mild degradative methods and hence should be of value in nucleotide sequence studies. Tamm *et al.* (155) find that they break down readily in alkali and suggest that this involves cyclisation at the free hydroxyl group at C_4 of the deoxyribose residue. It seems more likely [Todd (1)] that a β-elimination process should occur, activated by the carbonyl group, of the type discussed by Brown, Fried & Todd (121). Reduction in carbonyl activity should be effected by acetal formation with consequent change in hydrolytic behaviour toward the mechanism involving intermediate cyclic phosphate formation. Although no direct evidence for this view has been given, the requisite derivatives have been made and their hydrolyses studied. Lucy & Kent (156, 157) have prepared the ethyl mercaptal (XVII; R = Et) by treating DNA with ethylthiol and hydrochloric acid. Jones & Letham (158) form a similar derivative (XVII; R = $\cdot CH_2 \cdot CO_2H$), using thioglycollic acid in the absence of a strongly acidic catalyst. Both papers show that these materials are degraded profoundly by dilute alkali [compare however, Tamm & Chargaff (159)], yielding polynucleotides which, how-

ever, still contain sulphur. Although the evidence given does not indicate that pure materials were isolated, it does seem that further studies would be rewarding.

Seraidarian, Canzanelli & Rapport (160) discuss the changes which take place when nucleic acids are irradiated with ultraviolet light. The pyrimidine rings are most rapidly attacked, and ultraviolet absorption is reduced by one-sixth to one-third before any effect on internucleotidic linkages is noted. Christensen & Giese (161) make comparable observations. They also find that ozone attacks nucleic acids; the purine and pyrimidine residues are very sensitive to this reagent as would be expected. Smith (162) reports on the breakdown of DNA under deuteron bombardment, while Setlow & Doyle (163) describe the results of irradiating dry DNA with deuterons and electrons. Scholes & Weiss (164, 165) show that labile phosphate esters are produced by irradiation of nucleic acids by x-rays and suggest that oxidation at C_4 of the pentose moieties is involved. They consider (165) that slow hydrolysis of these labile ester groupings, together with the breakdown of products formed by attack on the heterocyclic ethylenic bonds, may contribute largely to the "after effect" (i.e., decrease in viscosity of nucleic acid solutions after cessation of irradiation) [cf. Conway (166)]. Butler & Conway (167) described the action of photochemically generated radicals from hydrogen peroxide on DNA and some simple model substances. They note that the action is remarkably similar to that of x-rays and find that all of the groupings in the molecule can be affected by this reagent.

Nucleases.—Until chemical degradative methods, for example, those based on the apurinic acids, can be refined sufficiently, it is necessary to rely on enzymatic hydrolysates as sources of oligonucleotides for structural studies. If it is accepted that the deoxyribonucleic acids and hence the polynucleotides derived from them, are linear molecules with a 3′,5′-internucleotidic linkage, it is to be expected that structural work will be confined in the main to sequence determination. Nucleotide order can be determined in the case of a di-deoxynucleotide, effectively by showing which residue carries the monoesterified phosphate [Sinsheimer & Koerner (168)], but methods applicable to larger molecules have yet to be devised. Sinsheimer (169) has made a careful study of the ion-exchange separation of pancreatic deoxyribonuclease digests of thymus and wheat germ DNA and has, thereby, purified the mono- and dinucleotide fractions. Over 95 per cent of the digests can be eluted from a 2 per cent cross-linked Dowex-1 resin. He finds that the mono- and dinucleotides represent 1/100 and 1/6, respectively, of the total digest. All the mono- and almost all of the possible dinucleotides are found to be present. In the latter category he confirms the observation of Markham & Smith (170) that a very much larger amount of the dinucleotide containing 5-methylcytosine and guanine is found than would be calculated on random distribution of 5-methylcytosine which is a minor base constituent. Although the mode of action of the enzyme is unknown, Sinsheimer points out that the results suggest that the enzyme may attack a particular

(although undetermined) internucleotidic linkage except when such a linkage is terminal in the chain fragment, in which case action may proceed slowly. This would give all possible di- and trinucleotides but limit the composition of tetra- and higher polynucleotides. It will be of some interest to know whether the dinucleotides isolated represent one or both of the possible isomers; this, in itself, would go some way to defining the specificity of the enzyme. Michelson & Todd (67) report that the enzyme does not affect the benzyl esters of deoxycytidine-3′ and -5′ phosphate and thymidine-5′ phosphate. This, however, is not surprising in view of the very small amounts of mononucleotides formed from DNA.

There is now good evidence that deoxyribonucleases exist with specificities differing from that of the pancreatic enzyme. Laskowski and coworkers (171) described the partial purification of deoxyribonuclease II from thymus. Evidence for different specificities of deoxyribonuclease I (from pancreas) and deoxyribonuclease II is based on ion-exchange elution patterns and paper chromatography of the di- and trinucleotide products, larger amounts of higher oligonucleotides being given by the latter enzyme (172, 173). Brawerman & Chargaff (234) find a deoxyribonuclease in germinating barley which hydrolyses DNA to give a "core" which is analytically different from that produced by pancreatic deoxyribonuclease. Maver & Greco (116) describe, briefly, the purification of a deoxyribonuclease from spleen, while Siebert *et al.* (174) demonstrate the presence of another deoxyribonuclease, pH optimum 5, in some mammalian organs.

Two urinary deoxyribonucleases with pH optima of 5.6 and 7 have been separated and their excretion under normal and postirradiative conditions have been studied [Kowlessar *et al.* (175); Koszalka *et al.* (176)].

Deoxyribonucleic acids.—Some studies of the preparation and properties of nucleoproteins have been recorded. Luck and co-workers (177, 178) described the isolation and composition of a nonfibrous deoxyribonucleohistone from liver and give sedimentation and electrophoretic data. The nucleohistone from thymus and the histone prepared from it have been studied [Shooter *et al.* (179); Butler *et al.* (180)]. Volkin (181) isolates a nucleoprotein from bacteriophage which shows a single component in the ultracentrifuge and which accounts for 25 per cent of the phage protein. Crampton *et al.* (182) report on the dissociation and reassociation of calf thymus deoxyribonucleoprotein in sodium chloride solutions. Jones & Marsh (183) have studied the deproteinisation of nucleoproteins by several methods, while Frick (184) has examined critically some of the commonly used methods for DNA preparation.

Deoxyribonucleoproteins can be fractionated by successive extraction with 0.6M sodium chloride solution. The DNA isolated from the various fractions have different base ratios, A+T/G+C varying from 0.76 to 1.31 [Lucy & Butler (185)]. Bendich (186) claims to demonstrate inhomogeneity in DNA. He isolates two fractions from rat tissues which differ in base composition and in which a significant departure from unity in the adenine/thy-

mine ratio is noted. Fractionation of calf liver DNA is achieved by chromatography on a substituted cellulose anion exchanger using neutral salt solutions for elution. This method resembles that used by Brown & Watson (187) in which a histone-coated keiselguhr column was employed.

Most of the recent papers devoted to the base compositions of deoxyribonucleic acids from diverse sources attest to the fact that the ratios purine /pyrimidine, adenine/thymine, and guanine/cytosine lie closely about unity [*inter alia*, Wyatt (188); Butler (189; Hurst *et al.* (190); Woodhouse (191); Uzman & Desoer (192)]. In this connection it is of interest that when grown in the presence of 5-halogeno-uracils several microorganisms and bacteriophages incorporate the foreign base into their DNA in very considerable amounts [Wacker *et al.* (193); Dunn & Smith (194); Zamenhof & Griboff (195)]. It is found (194, 195) that 5-bromouracil replaces part of the thymine quantitatively while the relative proportions of the other bases remain unchanged.

These analytical relationships, taken together, formed one of the bases for the macromolecular structure of DNA proposed by Watson & Crick (132). These authors have now given a detailed description of the structure together with an interesting account of the methods used in arriving at it [Crick & Watson (196); Crick (197)]. Briefly, it is proposed that in DNA there are two polynucleotide chains in the structural unit; these are wound helically round the same axis and run in opposite directions. The sugar-phosphate chain forms the helical backbone, diameter 20Å, and the bases lie inside the structure perpendicular to the long axis. Stability in the system depends on linkage between the chains through hydrogen bonds involving the base residues. Specific base-pairing is postulated whereby adenine is paired with thymine and guanine with cytosine (or derivatives). This accounts well for the observed base ratios and yet permits the wide variation in base composition found experimentally. The two chains in this structure are complementary, the nucleotide sequence in one determining that in the other. Such a system provides, in theory, a molecular basis for a genetic determinant [Crick & Watson (198)]. The process of replication (i.e., during cell division) has been discussed [Watson & Crick (199); Delbrück (200)], as has the means whereby the information contained in the arrangement of nucleotides in the double helix can be realized, for example, in determining the amino acid sequence during synthesis of specific proteins [Gamow (201)].

The x-ray crystallographic data on sodium deoxyribonucleate fibres finds its most ready interpretation in a two-strand helical structure [Franklin & Gosling (202)]. Of considerable significance is the fact that deoxyribonucleic acids from a variety of sources give the same x-ray diagram indicating that all are constituted on the same pattern [Wilkins, Stokes & Wilson (203)]. Jacobson *et al.* (204) study the hydration of DNA by proton magnetic resonance. Depending on water content, two modifications are distinguished which are reversibly interconvertible [Franklin & Gosling (202, 205)]. A crystalline structure exists at 75 per cent relative humidity while at and above 92 per

cent relative humidity a paracyrstalline form is observed, and this appears to be the one present in solution. The evidence points to a double helical structure for the latter modification with a pitch of 34Å and 10 nucleotide residues per turn of each strand. It thus corresponds closely to the model of Watson & Crick. Further detailed analysis should lead to more precise evaluation of the structural parameters, but present evidence suggests that the 2-strand helical conformation has passed beyond the stage of conjecture.

Irreversible structural alteration of systems constituted on the above lines should result from the action of agents which break (*a*) hydrogen bonds and (*b*) internucleotidic linkages. Several authors record changes in the electrometric titration curve, ultraviolet absorption, infrared spectrum, and viscosity when solutions of DNA are subjected to heating or ultrasonic vibration or to changes in pH to the acid or alkaline side or salt concentration [Laland *et al.* (206); Peacocke (207); Blout & Lenormant (208); Frick & Rosenberg (209); Thomas (210); Cavalieri & Stone (211); Blout & Asadourian (212)]. These effects are, in general, interpreted in terms of hydrogen bond fission. During hydrolysis of DNA by deoxyribonuclease similar effects are noted, i.e., increased ultraviolet absorption, changes in infrared spectrum and in dye binding capacity [*inter alia*, Laland *et al.* (206); Blout & Lenormant (208); Kurnick (213)]. In this case hydrolysis of the internucleotidic linkages must be followed by spontaneous hydrogen bond rupture, leading to the observed spectral changes. Zamenhof *et al.* (214, 215) study the effect of many of the above agents on several deoxyribonucleic acids and in particular compare loss of biological activity of bacterial transforming factors with physical and chemical changes.

Dekker & Schachman (216) assess much previous data of the kind recorded above and propose a modification of the Watson & Crick model. They suggest that the individual polynucleotide chains instead of being continuous are short, being interrupted about every 50 nucleotide residues with breaks in the two strands staggered relative to one another. They study the early stages of deoxyribonuclease action at very low concentrations of the enzyme and are led tentatively to conclude that the number of bonds which have to be broken in order to reduce the viscosity significantly is much less than would be expected for a double helix composed of continuous strands. In contrast to this, and in support of the original Watson & Crick formulation, is the claim, as yet unbacked by experimental details, that in solutions of 2 to 4M urea the molecular weight of DNA is halved [Stacey & Alexander (217)]. Dekker & Schachman quote, in support of their proposal, recent titration studies by Lee & Peacocke (218) which indicate one secondary phosphoryl dissociation per 20 to 30 nucleotide residues. This suggestion seems more satisfactory than that proposed by Lee & Peacocke in which branching through phosphotriester groupings was invoked to account for the titration results. Mere heating of neutral DNA solutions reduces their viscosity [Zamenhof *et al.* (215)] but would not be expected to break phosphotriester bonds, whereas 0.1*N* alkali, which should lead to hydrolysis,

does not yield more than traces of dialysable material [cf. Frick (219)]. Thus there is little evidence for short branches; ramified structures with long branches are incompatible with the physical evidence, including electron microscopy [Kahler & Lloyd (220)], which leads uniformly to the opinion that DNA is in the form of long rod-like molecules.

A number of papers deal with the form and dimensions of DNA molecules in solution. Sadron (221) has contributed a critical review of the methods at present used in these studies. A detailed examination of recent measurements and their interpretation cannot be given here, but a considerable degree of unamimity appears to have been reached among workers in this field. The DNA molecule appears to be highly asymmetric with an axial ratio greater than 300. Much of the data from light-scattering, flow-birefringence, viscometric and sedimentation studies can be accomodated on the assumption the molecule is in the form of a slightly flexible rod of diameter about 20Å and a contour length 0.6 to 2.0μ which decreases in solutions of increasing salt concentration [Reichmann *et al.* (222); Goldstein & Reichmann (223); Rowen *et al.* (224, 225); Peacocke & Schachman (226); Jordan & Porter (227); see also, Anderson (228)]. These properties appear to be consistent with the helical model discussed above. Rowen & Norman (225) comment on the difficulty of accomodating these molecules into cell structures, e.g., the Feulgen-positive body of *Escherichia coli* which has a diameter of 0.6μ and consider that coiling or folding must occur. The discontinuities in the Dekker & Schachman model may provide the means for folding.

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WATER-SOLUBLE VITAMINS, PART I[1]
(VITAMIN B_{12}, FOLIC ACID, CHOLINE, AND PARA-AMINOBENZOIC ACID)

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This review is intended to cover the pertinent literature available to the authors up to about December 15, 1954, and published since the last review by Lester Smith (1). Because of the large number of publications in this area, coverage of every paper on this subject could not be given. Rather, the authors have selected what appear to be the more important papers and have expanded certain sections at the expense of others. No special effort has been made to establish priority.

VITAMIN B_{12} (COBALAMIN) AND RELATED FACTORS

Although it has been seven years since B_{12} was isolated, the number of papers on this subject is still increasing. Over 600 papers on B_{12} appeared in 1953 and 1954 since the last review (1). Interest seems to be centered on clinical uses of this vitamin in a variety of diseases. Several reviews or monographs on B_{12} have appeared recently which cover the entire field to 1953 (1 to 5).

Nomenclature.—Throughout this review the related forms of B_{12} with animal (vertebrate) activity are collectively called the B_{12} group, or cobalamin ("true" B_{12} group); whereas B_{12}-related compounds with growth promoting activity only for lower forms of life are designated as members of the pseudo B_{12} group and are given specific names where available. These latter compounds are not properly classified as "vitamins" in the accepted sense, although they are closely related.

CHEMISTRY OF B_{12} AND PSEUDO B_{12} COMPOUNDS

Chemistry of B_{12}.—Because of the complexity of the molecule, relatively little progress has been made toward the determination of the structure of B_{12} (see reviews 1, 5, 6, 7). Folkers and co-workers (7, 8, 9) have tentatively determined that the phosphate group is in the 3′-position on the α-ribazole rather than in the 2′-position. These workers prepared D-1-amino-2-propanol from the isomeric mixture by a relatively simple method (9). Heyl *et al.* (10) and Johnson *et al.* (11) studied the synthesis of various 1-glycosides of

[1] The following abbreviations are used in this chapter: ADP for adenosinediphosphate; ATP for adenosinetriphosphate; B_{12} for vitamin B_{12}; pseudo B_{12} for pseudovitamin B_{12}; CF for citrovorum factor; DNA for deoxypentosenucleic acid; DPN for diphosphopyridine nucleotide; FA for folic acid; IF for intrinsic factor; PABA for *p*-aminobenzoic acid; PGA for pteroylglutamic acid; RNA for pentosenucleic acid.

5,6-dimethylbenzimidazole. This information will be useful in exploring synthetic methods of B_{12} preparation.

A preliminary report, requiring confirmation, gives an allocation of the 13 nitrogen atoms of B_{12b} [Diehl and co-workers (12, 13)]. Five atoms of nitrogen were found as NH_3 in an acid hydrolysate (from amide groups), two atoms in 5,6-dimethylbenzimidazole, four in the red acid fragment, and two in 1-amino-2-propanol (or alternatively, one of these two in a small unknown nitrogen compound). These authors provided evidence that at least some of the acid amide groups were "located sufficiently closely to permit the formation of cyclic anhydrides or imides" (14). Upon hydrolysis with acid, it was found that three of the amide groups were released at a more rapid rate than the other two (15). Jaselskis & Diehl (16) have given results of further studies on the polarography of B_{12b} ("B_{12a}") and B_{12r} (catalytically hydrogenated B_{12}, which probably does not occur in nature). It was found that B_{12b}, unlike B_{12}, undergoes two one-electron reductions corresponding to changes in valence of the cobalt from 3 to 2 and from 2 to 1. B_{12r}, in which the cobalt is bivalent, showed one anodic and one cathodic wave agreeing in position and height with the wave of B_{12}.

Brink *et al.* (17) and Cannon *et al.* (18) recently offered important data, obtained from x-ray analysis, which indicates that the nucleotide-free degradation product of B_{12} ($C_{47}H_{66}O_{16}N_8CoCl$) contains a new type of 5 membered ring system, probably a modified porphyrin structure. Other x-ray crystallographic studies on B_{12} provided only very little new information (19, 20).

Electrophoretic studies of B_{12} (21, 22) showed that the B_{12} molecule moved toward the anode when the pH was 2.95 or higher, but toward the cathode at pH 1.1 to 1.8. The isoelectric point appeared to be near pH 1.9. Also, the presence of both weak acidic and weak basic groups in B_{12} was confirmed. Extending and confirming earlier work of others, it was shown (22) that B_{12b} (hydroxocobalamin and aquocobalamin) existed in equilibrium, with the aquo-form dominating below pH 8.1 and the hydroxo-form above pH 8.1. Above pH 11.2 it appeared possible that a"di-cobalamin" was formed in which two B_{12} molecules shared an OH^- group.

A new compound of the B_{12} series with anti-pernicious anemia activity has been obtained from bacterial sources by Fantes & O'Callaghan (23) and Ford *et al.* (24). This analogue of B_{12} (desdimethyl B_{12}) contains benzimidazole rather than the 5,6-dimethyl derivative and appears to be fully as active as B_{12} (see page 342 for further details).

Bradley *et al.* (25) have described the preparation of labeled B_{12} containing radioactive isotope of Co^{58} rather than Co^{60}, the form available in the past. The authors demonstrated its usefulness in a study with a patient with pernicious anemia and recommended its use in the future instead of B_{12} with Co^{60}. Co^{58} has the advantage of increased safety because of a much shorter half life (72 days) compared with Co^{60} (5 years).

Stability of B_{12}.—Most of the studies this past year on the stability of

B_{12} have been concerned with pharmaceutical preparations of this vitamin. In general, it appears that B_{12} may be considered as one of the more stable vitamins. When properly handled it retains its effectiveness under a wide variety of temperature and storage conditions (26 to 29; also see reviews 5, 30). Conversion to B_{12b} may occur during storage, but this form is also active (27). B_{12} is stable when mixed in solution with most other vitamins if properly buffered, but reducing agents should be avoided (30). B_{12} withstands high drying temperatures in fishery by-products under conditions which lead to a loss of nicotinic acid (31). Unfortunately, most of the tests on B_{12} stability have been conducted with methods which may not adequately test activity for higher animals.

Commercial production of B_{12}.—The demand for crystalline B_{12} and B_{12} concentrates for pharmaceutical and feed uses is steadily growing. It has been stated that a total of 94 pounds of B_{12} at a value of over 5 million dollars was produced in 1952 (32). The cost of B_{12} is gradually decreasing. At the time of this writing bulk crystalline B_{12} was 25¢ a mg. and B_{12} in concentrate form for feed use was approximately 5¢ a mg. All B_{12} available today is from microbiological fermentation sources; none is made synthetically (see reviews 32, 33, and section on distribution of B_{12}). A method for preparing B_{12} concentrates from *Streptomyces olivaceus*, at an estimated cost of 2.7¢ per mg. of B_{12}, has been described (32, 34, 35). A number of United States and foreign patents on B_{12} production, concentration, purification, recovery, crystallization, and stabilization were granted in 1953 and 1954 (36, 37).

Standard preparations of cystalline B_{12} made in Britain and in America were found to have equal microbiological activity (38). However, caution must be taken in assuming that preparations of crystalline B_{12} are pure unless rigid standards are maintained. Chromatography, electrophoresis, and ionophoresis have demonstrated a number of impurities (compounds structurally related to B_{12}) to be present in certain preparations of B_{12} or B_{12b} (1, 39).

Chemistry and biochemistry of pseudo B_{12} group.—It is well accepted that a number of B_{12} analogues exist in nature which are not active for animal growth or for pernicious anemia but which have B_{12} activity for certain microorganisms. These compounds have been called by a variety of names, including Factor A, Factor B, Factor C, vitamins B_{12m}, B_{12f}, pseudo B_{12}, etc. A few of these names now are known to have been assigned to mixtures of compounds and such names are now obsolete (1). The confusion is gradually being cleared, however, through the use of improved methods of separation.

Ford & Porter (40) have published details of their earlier studies on Factors A, B, and C, obtained from bovine gastrointestinal contents and feces, and have compared microbiological activities of these factors. Factor A, now separated chemically, is apparently the most abundant B_{12}-like compound in gastrointestinal contents. Factor B, originally isolated by Ford & Porter (40, 41), appears to be the parent substance in the formation

of other compounds which may have B_{12} activity (1, 42). Factor B is similar to B_{12} except that it does not have the nucleotide group and is devoid of animal activity. Hydrolysis of either B_{12}, pseudo B_{12}, or Factor A with concentrated HCl removes the specific nucleotide group, thus giving rise to Factor B in each instance (42).

Ford and co-workers (24, 43) have produced a number of compounds containing Factor B by guiding the synthetic activities of bacterial mutants. Factor B was added to the medium along with various compounds, including benzimidazole, 5,6-dichlorobenzimidazole, 5-methylbenzimidazole, 5-aminobenzimidazole, 5-nitrobenzimidazole, 4-chloro-1,2-benztriazole, benzthiazole, 2,8-dichloroadenine, and 2,6-diaminopurine. This has resulted in the formation of new B_{12}-like compounds with varying degrees of pseudo B_{12} activity for *Escherichia coli*, *Lactobacillus leichmannii*, and, in some cases, for the protozoan *Ochromonas malhamensis*. The activity of some of these compounds for *Ochromonas* indicates that they may have true B_{12} activity; however, there is as yet no evidence that any of these compounds normally occur in nature.

Using another technique Fantes & O'Callaghan (23) also have isolated what seems to be the B_{12} analogue containing benzimidazole by adding *o*-phenylenediamine to the medium for an undisclosed organism (*Streptomyces griseus*?). The compound (desdimethyl B_{12}) had a similar absorption spectrum to B_{12} but appeared to be 2 to 4 times as active in the *E. coli* plate assay and 1.8 times as active for *L. leichmannii*. Of real interest was the report that this compound was at least as active as B_{12} in three patients with pernicious anemia (23). Of further interest is the finding that the addition of 1,2-diamino-4,5-dimethylbenzene to the medium inhibited the biosynthesis of this new factor in the presence of *o*-phenylenediamine.

The presence of 2-methyladenine in Factor A (pseudo B_{12d}) was established independently by Brown & Lester Smith (44) and by Dion *et al.* (45). Furthermore, preliminary reports indicate the presence of hypoxanthine in deaminated pseudo B_{12} (*Factor G*) and of 2-methylhypoxanthine in deaminated Factor A (44, 46). Both Factors G and H have been obtained from pig manure. According to these investigators, this is the first time that the purines 2-methyladenine and 2-methylhypoxanthine have been found in nature.

Pfiffner *et al.* (46) have described other cyano-pigments including pseudo B_{12b} (containing adenine) and pseudo B_{12f}. Pseudo B_{12f} contains 2-methyladenine but differs from Factor A in its index of refraction. Several other similar compounds, which had no microbiological activity, were also found.

Ericson and co-workers (39, 47) confirmed the occurrence of many microbiologically active forms of B_{12} in nature. They described several new forms including one from seaweed called "vitamin B_{12a}" (which actually may be a mixture of factors). It is unfortunate that the term vitamin was used in tentatively naming this factor or factors because no animal activity was demonstrated. Other investigators (48, 49) have also confirmed the presence,

in natural sources, of a number of microbiologically active B_{12} entities. In one of these studies (49) further purification steps would be necessary to prove the reported anti-pernicious anemia activity of "B_{12m}."

Coates *et al.* (50) confirmed the lack of animal (chick) activity of concentrates of pseudo B_{12}. Also, the inactivity of pseudo B_{12} for the pig (51)

TABLE I

SUMMARY OF PRINCIPAL NATURALLY OCCURRING COMPOUNDS WITH B_{12} OR PSEUDO B_{12} ACTIVITY AND THEIR ANIMAL OR MICROBIOLOGICAL ACTIVITY*

Compound†	Purine or other group present	Activity for Vertebrates	Activity for Microorganisms‡			
			L. leichmannii	*E. coli*	*Euglena*	*Ochromonas*
1. Vitamin (B_{12}) cyanocobalamin)	5,6-dimethylbenzimidazole	+	+	+	+	+
2. Vitamin B_{12} (hydroxo- or aquocobalamin)	5,6-dimethylbenzimidazole	+	±	±	+	+
3. Vitamin B_{12} analogue (desdimethyl B_{12})	benzimidazole	+?	++	++		+
4. Factor A (pseudo B_{12d})	2-methyladenine	−	±	+	+	−
5. Factor B (parent compound)	none	−	−	++	−	−
6. Factor C (C_1 and C_2)		−	±	++	+	−
7. Pseudo B_{12}	adenine	−	++?	+	±	−
8. Pseudo B_{12b}	adenine	−	+		-?	
9. Factor G (deaminated pseudo B_{12})	hypoxanthine	−	±	+		
10. Factor H (deaminated Factor A)	2-methylhypoxanthine	−	±	+		
11. Pseudo B_{12f}	2-methyladenine	−	±			
12. "Vitamin" B_{12s} (a mixture? Factor C?)		−		+		

* See text for references.

† Other names have been postulated but are now obsolete because they have been shown to be mixtures of some of the above more purified fractions. The authors were unable to find references to Factors D, E, or F.

‡ Only rough approximations of activity compared with B_{12} are shown. The comparative potencies are still difficult to assess and there is not always agreement between laboratories. Often comparisons differ even by method of assay with the same organism. (± indicates low activity; ? denotes questionable activity; and a blank space indicates no information available)

and for the cow (52) was demonstrated for the first time by direct feeding experiments.

Table I gives a general list of B_{12} and related factors summarizing existing information on microbial and animal activity.

B_{12} ASSAY, MICROBIOLOGICAL STUDIES, AND DISTRIBUTION

Of many suggested chemical or microbiological methods for B_{12} determination in crude materials, only the recently proposed *Ochromonas* assay

and the isotope dilution method appear to give results that agree with animal activity. Most methods in routine use today do not distinguish between microbial and animal activity. For example, Chaiet *et al.* (53) recently compared several widely used microbiological assays with spectrophotometric methods for B_{12} in commercial products used in feed manufacturing. The results were also compared with chick assay values. It was found that none of the routine methods differentiated between B_{12} and pseudo B_{12}. In fact, one commercial B_{12} feed supplement was found which was devoid of true B_{12} activity, and the values from other supplements correlated very poorly with values from chick assays.

Chemical or physical methods.—B_{12} may be satisfactorily determined by chemical or physical means after purification from crude sources. Fisher (54) described a rapid spectrophotometric method which when applied to fresh and dried bacterial cells is "simple to operate, and is much faster and more accurate than bioassay." B_{12} was extracted by benzyl or *n*-propyl alcohols before measurement. The working time of the method was only 1 to 2 hr. Although spectrophotometric methods appear to be very useful for control and standardization of B_{12} concentrates, they may give rise to misleading results as far as true B_{12} activity is concerned (53, 55). Several chemical methods for estimation of B_{12} in liver extracts have been suggested (27, 56, 57, 58). They appear satisfactory as adjuncts to other methods.

An interesting radioactive tracer technique for B_{12} compounds in pharmaceutical or fermentation products was described by Bacher *et al.* (59). The method requires purification of B_{12} followed by spectrophotometric determination. Unlike most other chemical or bacterial methods, this method differentiates between pseudo B_{12} and true B_{12} and appears very promising. Chemical methods were suggested for distinguishing between B_{12} and B_{12b} in pure solutions (60) or in liver extracts (27).

Animal assays.—A previously suggested assay for B_{12}, using mice made anemic with phenylhydrazine, showed poor agreement between results obtained with crystalline B_{12} and liver extracts of known B_{12} content (61). It was suggested that liver extract may contain other hematopoietic substances. Results of chick assays showed only fair agreement with microbiological assay results, depending on the material tested (50, 62). Assays made with chicks gave markedly lower results for gastrointestinal contents and feces than did any of the microbial tests used, indicating again the preponderance of pseudo B_{12} in gastrointestinal contents and the importance of the problem of specificity in test procedures (50). An improved assay with normal chicks for B_{12} activity is suggested by the large differences in weight obtained with B_{12} when added to a corn-soybean meal ration containing 20 per cent of added fat [Spivey *et al.* (63)]. With such an assay, allowances must be made for methionine which under these conditions spares B_{12} (64). A rat assay for B_{12} and for unidentified factors has been outlined by Sherman & Schaefer (65). Also a test for B_{12} in the rabbit has been reported based on the counteraction with this vitamin of lead-induced anemia (66).

Microbiological assays.—The availability of recent reviews on this subject obviates the need for a lengthy discussion (67, 68, 69). The use of *L. leichmannii* 7830 for B_{12} assay remains popular even though it is not specific. Various recent refinements and improvements in the assay (70 to 78) lead to increased consistency, improved reliability of results, and better methods of liberation of B_{12}. A 16 hr. assay with this organism has been recommended for routine clinical use on serum in cases of suspected pernicious anemia (78). Small amounts of histamine appeared to have a synergistic action with B_{12} in a *L. leichmannii* 4797 assay (79, 80), and the presence in the media of large amounts of ascorbic acid and certain trace minerals (as in pharmaceutical preparations) may cause destruction of B_{12} (81). Skeggs *et al.* (82) have studied additional purine, pyrimidine, and B_{12} interrelationships in this organism.

Refinements with the use of mutant *E. coli* 113–3 for B_{12} assay have been suggested (83 to 89). Assays with this organism are rapid (15 to 18 hr.) and appear to be as accurate and reliable as those made with *L. leichmannii*. *E. coli* responds to methionine in the absence of B_{12}, but such sources of error can be partially eliminated (85, 86, 87, 89). *E. coli* mutants grown in the absence of B_{12}, also respond to certain unidentified factors, possibly of peptide nature (90), and to a synthetic cobalt-nucleic acid complex of unknown structure $(C_{38}H_{48}N_{18}O_{23}P_4)_nCo$ (91).

Details of the first microbiological B_{12} assay (with *Lactobacillus lactis*), used successfully by Merck & Co. for the isolation of B_{12}, have been published [Shorb & Briggs (92)]. Although *L. lactis* is still occasionally used for B_{12} assay (27, 93), more reliable methods are now available. Berman *et al.* (94) suggested that *Lactobacillus acidophilus* 832 was useful for the differential assay of pseudo B_{12} compounds because of the high activity of pseudo B_{12} for this organism.

Improvements in protozoan assays for B_{12} have also been reported. Such assays are more specific than bacterial assays for B_{12} activity (67, 68); however, they have a disadvantage of requiring longer incubation times (as much as 7 to 18 days in some cases). Several recent papers describing tests with *Euglena gracilis* have appeared (95 to 99). The growth of *Euglena* is now known to be stimulated by pseudo B_{12} equally as well as by B_{12} itself. However, ribazole and benzimidazole, active for *E. coli*, are relatively inactive for *Euglena* (69, 100). Several rather unique methods for evaluating the amount of growth of *Euglena* in a B_{12} assay have been suggested (97, 98, 99).

As yet, there has not been sufficient time to fully evaluate and confirm the high specificity of the protozoan *O. malhamensis* for true vitamin forms of B_{12} (see Table I) as demonstrated by Ford (101). Recently, Gregory (102) compared results obtained with this organism with results from assays with *L. leichmannii* and *E. coli*. Also, it is known that three different strains of *Ochromonas* require B_{12} (103). More studies with this organism are needed because of the apparently excellent correlation of results with animal activ-

ity. The use of *Poteriochromonas stipitata* for a 5 to 7 day assay of true B_{12} activity has been described by Barber *et al.* (104) and compared with other microbial methods of assay (except with *Ochromonas*). Many other investigators have also compared different methods of assay. These include Scheid & Schweigert (105), who studied the B_{12} content of meats with *L. leichmannii* 327 and *Euglena*; Dawbarn & Hine (106), who compared the *L. leichmannii* 313 tube method with an *E. coli* plate assay; and others who have compared various methods (27, 50, 53, 54, 62, 81, 83, 85, 88, 89, 107).

In summary, the method of choice for B_{12} depends upon the purpose of the assay and the material to be analyzed. Each type of assay offers certain advantages and disadvantages. The use of a combination of methods seems to be advisable for best interpretation of results when assaying a variety of crude products. Of the microbiological methods, the *Ochromonas* assay is best suited for determination of true B_{12} activity; however, the bacteriological assays are more practical for routine purposes when the presence of pseudo B_{12} is not a complicating factor.

Other microbiological studies.—Dubnoff (108) has compared B_{12} requirements of several variants of *E. coli* mutant 113–3 and found marked differences. Homocysteine could replace the requirement for B_{12} in the variant with the lowest B_{12} requirement (0.018 mμg. B_{12}/ml.) while intermediate variants used homocysteine only if supplemented with small amounts of B_{12} or with PABA[1] and dimethyl-β-propiothetin (a methylating agent). Mutants with the highest B_{12} requirement (0.12 mμg. B_{12}/ml.) grew only with B_{12} or methionine.

An interesting depression of growth of *Mycobacterium tuberculosis* H37RV caused by 5,6-dimethylbenzimidazole and 1,2-dimethyl-4,5-diaminobenzene led Hallinger *et al.* (109) to believe that the compounds were acting as antagonists. Evidence was offered that *M. tuberculosis* produces B_{12} normally, which could conceivably explain the reported low incidence of pernicious anemia in patients with tuberculosis (109). Clinical trials with these "antagonists" in patients with tuberculosis would be interesting.

The need for B_{12} has been reported this past year for *Stichococcus* sp. (110), *Gymnodinium splendens* (111), *Peridinium* sp., *Cyanophora paradoxa*, *Synura* sp., a marine *Gyrodinium* (112), three additional strains of chrysomonads [*Monochrysis*, *Prymnesium*, and *Syrocosphaera* (113)], and for over 100 minor strains of *Euglena* (100). Growth of *Saccharomyces cerevisiae* was stimulated by B_{12}, but similar results were obtained with an equivalent amount of cobalt (114). Therefore, B_{12} does not appear essential for this organism or for any yeast, as far as is known. An interesting symbiotic relationship between *L. leichmannii* and *L. citrovorum* has been reported (115) in which the two organisms grew normally when raised together in a medium devoid of B_{12} and CF.[1] When grown separately in this medium no growth occurred.

Distribution.—B_{12} is unique among the vitamins because it is synthesized in nature only by certain microorganisms; chiefly, if not solely, by bacteria

and actinomycetes [reviewed by Darken (33)]. A possible source of B_{12}, which would be an exception to this statement if confirmed, is certain spontaneous mammary cancer cells in mice [Woolley (116, 117)]. Tissues of B_{12} deficient mice with mammary cancer contained larger amounts of B_{12} than tissues from nontumorous mice; however, the evidence offered is not too convincing, nor is there proof that the B_{12} was derived from the cancer cells rather than from other sources (e.g., microbial infection or intestinal synthesis). The presence of B_{12} in higher plants or in normal animal tissues is only as a result of the wide distribution in nature (in soils, water, gastrointestinal tract, etc.) of microorganisms, the original source of B_{12}. Higher plants, such as alfalfa, grass, corn, soybean, etc., contain no true B_{12} except fortuitous amounts absorbed from the environment. For instance, alfalfa meal was found, by chick assay, to be devoid of B_{12} activity (118).

Ericson & Lewis (47) reported that contrary to previously accepted ideas, algae did not have the ability to synthesize B_{12}. Instead, they found that closely associated bacteria produced the vitamin with eventual absorption and concentration by the algae. Thirty-four partially identified cultures of bacteria isolated from seaweed were able to synthesize B_{12} (especially well when grown symbiotically). As a result of the good utilization of this B_{12} from bacterial sources, algae (seaweed) is a rich source of B_{12}, as rich as the best known animal products. At least 25 species of marine algae are known to contain B_{12} in varying amounts (47, 119, 120). Of these, the green types of algae were richer in B_{12} than red or brown types. Other workers have also reported the presence of B_{12} in various species of seaweed (121), perhaps sufficient to serve as a practical, low cost source of B_{12} for poultry feed (122).

A large number of bacteria and actinomycetes contain B_{12} as a result of their synthetic ability. This past year reports have appeared confirming or showing for the first time the presence of B_{12} in *Streptomyces olivaceus* (32, 34, 35), *Serratia marcescens* (123), *Bacillus megatherium* (124, 125), *Aerobacter aerogenes*, *E. coli*, *Proteus vulgaris*, *Micrococcus roseus*, many strains of *Streptomyces*, and other organisms (125, 126). Also Levin *et al.* (127) found that certain *Rhisobia* (root nodule bacteria) synthesized B_{12} and that the concentration of B_{12} in pink nodules on alfalfa roots was 4 times greater than in white nodules. Interesting metabolic theories could be postulated from this fact but, as yet, there is no evidence that the alfalfa plant utilizes B_{12}.

It is known that, as a result of microbial growth, sewage is a very rich source of B_{12}. In fact, production of B_{12} concentrates for feed use from sewage sludge has been accomplished on a pilot plant basis (128, 129, 130). Dried sewage sludge serves as a good source of B_{12} for baby pigs without harmful effect when fed at a level of 2 per cent of the ration (131).

Values have become available for the B_{12} content of cow's milk (102, 132 to 135, reviewed in 136), human milk (102, 132, 134, 135, 137), buffalo milk (134), sow's milk (102, 135), sheep milk (102, 138, 139), and the milk of the goat and rat (102), for the effect of diet on the B_{12} content of cow's

milk (133), and for the effect of stage of lactation (138) and of change in diet (139) on the B_{12} content of sheep milk. Figures have appeared for the B_{12} content of the blood or serum of various animals including man, rabbit, goat, sheep, horse, guinea pig, ox, pig, dog, cow, chicken, and alligator (139 to 148) (for other references to man see later section); for the content of B_{12} in certain organs of the sheep (105, 106), rat (liver) (149, 150, 151), man (skin) (152), pig (105, 153, 154, 155), fish (156), cow (105, 157, 158), chicken (72, 159 to 162), rabbit (163); for B_{12} values in urine of man (137, 142, 143), sheep (164), and rabbit (163); for feces or gastrointestinal contents of the fowl (50, 72, 104, 161, 162, 165, 166), rat (50, 151), cow (50, 104, 157), rabbit (163), pig (154, 155), sheep (106, 164), and man (104, 167, 168); for the B_{12} content of chicks (145) and eggs (72, 145, 159, 161); of various fish by-products (31, 50, 169, 170); of various animal livers (70); and of miscellaneous materials including seawater (47), clams (121), and clam juice (104), and of distilled water (100). The latter was reported to contain up to 2.0 $\mu\mu$g. of B_{12} per ml. when kept under nonsterile conditions, whereas freshly distilled water was free of B_{12}.

Most of the values for B_{12} available in the literature must be considered as preliminary in light of newer knowledge of assay methods, of chemistry, and of biological activities of B_{12} and pseudo B_{12}. Information on the true B_{12} content of foods and feed is urgently needed.

Biochemical Function

Although as yet no enzyme or coenzyme containing B_{12} has been found, it is known that B_{12} is concerned in the metabolism of one-carbon fragments (1). Specifically, B_{12} aids in the *de novo* synthesis of labile methyl groups. Other important possible functions of B_{12} include its role in —SH metabolism and perhaps an indirect role in carbohydrate, fat, and protein metabolism. The exact mechanism of these reactions is unknown. In fact, the apparent absence of B_{12} in higher plants (see previous discussion) suggests that, unlike most other B vitamins, B_{12} is not an indispensable constituent or metabolite for all living cells. Thus, a proposed definition of B vitamins by Williams *et al.* (171) ("organic substances which act catalytically in all living cells" etc.) would have to be modified. Also, postulated theories for the metabolic action of B_{12} in transmethylation, nucleic acid synthesis, metabolism of one-carbon fragments, etc., must take into account the fact that cells of higher plants appear to be able to carry out many of these reactions without B_{12}. During this past year the following bits of information of B_{12} function became available (see reviews 5, 172).

General.—Further evidence for a possible metabolic relationship between coenzyme A and B_{12} was suggested by Boxer *et al.* (173, 174). They reported a 5-fold increase in coenzyme A concentration of the liver and a 2½-fold increase in the kidney of B_{12}-deficient chicks. Similar changes were found in rat liver (174). A B_{12}-coenzyme A relationship suggests possible effects of B_{12} on —S-S— groups as has been suggested previously (1). In this connection,

Register (175) found a decrease in total nonprotein sulfhydryl groups in the liver and blood of B_{12}-deficient rats. This decrease was accounted for by glutathione rather than by ergothioneine. Dubnoff & Bartron (176, 177) have described a "B_{12} glutathione" system in enzyme extracts of *E. coli* mutant 113–3 in which an effect of B_{12} on the oxidation of a variety of substrates is not obtained unless glutathione is present. Cyanide ions have a marked enhancing effect on this system, but details of the experiments are not yet available. If B_{12} does function in glutathione and coenzyme A metabolism, this could conceivably offer a mechanism for the observed relationship of B_{12} to the metabolism of fats and carboyhdrates, further evidence for which has been presented this year by Ling & Chow (178, 179, 180) and others (63, 181, 182). It is known that glutathione as well as coenzyme A plays an important role in enzyme systems concerned with fat and carbohydrate metabolism (see references in 175, 178). As one further step in this direction, the suggested influence of B_{12} on ribose formation in erythrocytes could explain the known effect of B_{12} in synthesis of nucleosides or nucleic acid, as has been suggested (180).

Additional pieces of evidence have been presented this past year for the role of B_{12} in nucleoside or nucleic acid formation in studies made with microorganisms (183), with spinal motor root cells of guinea pigs (184), with livers of rats subjected to partial hepatectomy (185), with livers of hyperthyroid rats (186), and with bone marrows of patients with megaloblastic anemia (187).

Edwards & Johnson (188) obtained evidence suggesting that B_{12} and methionine are involved in the formation of enzyme systems which contain riboflavin and nicotinic acid.

Amino acid metabolism.—Interest continues in the possible effect of B_{12} deficiency on amino acid metabolism. Increased blood levels of amino acids in the chick, formerly attributed to a deficiency of B_{12} (189), may be attributable to lowered feed consumption or inanition, as shown by more recent experiments (160, 190). However, B_{12} still appears to function as "a moderator of fasting effects on blood levels of free amino acids" when B_{12} is given to the chick in amounts larger than required for maximal growth (160). Hsu *et al.* (191) showed that B_{12} functions in nitrogen metabolism in the chick embryo. Richardson *et al.* (192) found that the level of five amino acids decreased significantly in the plasma of B_{12}-deficient chicks, but results with paired fed controls were not reported. A possible function of B_{12} in tyrosine metabolism in vitamin C-deficient guinea pigs was indicated by Wu & Sealock (193). In humans with pernicious anemia, amino aciduria may occur (194). Other relationships between B_{12} and amino acid metabolism are known but, as yet, no direct relationship has been proven.

Methyl group relationships.—Studies made this past year on this phase of B_{12} metabolism have confirmed and extended previous observations. Studies with the baby pig by Johnson and co-workers confirm that B_{12} is required for the incorporation of the α-carbon of glycine into the methyl

group of choline, whereas B_{12} has no effect on the formation of serine or aminoethanol from glycine (195, 196). Further confirmation of the *de novo* synthesis of methyl groups has been obtained in experiments with chicks (197). The role of B_{12} in choline formation from glycine may partially explain the lipotropic effect obtained with B_{12} in rats on low choline, low protein diets (198, 199, 200). B_{12} does not seem to be involved in transmethylation as shown in experiments made with the chick (197), with the baby pig (201), or with pig liver homogenates (202) which confirms previous conclusions (1).

Miscellaneous.—Further studies have appeared showing the possible need of B_{12} in the conversion of PGA[1] to CF (159, 203). This transformation may be related to the apparent role of B_{12} in metabolism of single carbon fragments. Ballentine (204) has suggested that B_{12} may have a role in the exchange of cobalt from a common ionic cobalt pool in *Neurospora* into stable protein complexes, but confirmatory evidence is needed for this theory. A possible role of B_{12} as an oxygen carrier with the ability to catalytically oxidize iodide is suggested by the chemical studies made by Brierly *et al.* (205); however, biological systems have not been studied in this connection.

Therapeutic Uses and Intrinsic Factor

General human nutrition.—According to the Food and Nutrition Board (206) there is still no conclusive evidence that a healthy "normal" adult requires a dietary source of B_{12}. Recent publications do not change this concept. If B_{12} is required, it appears likely that the amount synthesized in the intestinal tract and absorbed through the intestinal wall is adequate under normal conditions. However, carefully conducted long range studies with normal individuals on B_{12}-deficient diets (vegetarians, for example) are urgently needed to study adult requirements.

In spite of the absence of conclusive evidence, there is a growing number of reports, often questionable, that B_{12} supplementation of diets for infants or underweight children may result in small but beneficial weight gains, increased heights, or improved appetites (207 to 212). Unfortunately, often the experiments have been poorly controlled (such as lack of placebos), conducted with small numbers of individuals, or have made use of B_{12} of undisclosed purity, rather than crystalline B_{12}. Additional studies with children are needed including those publications showing only negative results, before final conclusions can be drawn regarding the effectiveness of B_{12}.

In a well controlled experiment by O'Brien (213), 47 per cent of a group of normal adults injected with B_{12} stated that they felt better; however, so did an equal percentage of controls injected with saline. It is often this latter control that is omitted in reported experiments. Bayne & Boger (214) also reported no significant change in 44 healthy adult females given B_{12} and receiving normal diets. In a study with 150 partial vegetarians receiving diets almost free of B_{12}, Wokes (215) has reported macrocytosis and low B_{12} blood values in some of the subjects, although as yet no details of the experiment are available. Heinrich (137) reported that 20 per cent of a

group of supposedly normal pregnant and lactating women had low serum and urine levels of B_{12} and suggested an increased B_{12} requirement during pregnancy and lactation of 5 to 10 μg./day. It is difficult to interpret these data because clinical signs of a deficiency were not reported. Normal older individuals appear less able to absorb B_{12} and have a lowered B_{12} level in blood according to Chow and associates (216, 217, 218). Whether or not this indicates a higher requirement for B_{12} by older individuals is not known.

A number of other studies have been made on factors affecting the B_{12} content of the blood, which under normal conditions ranges from 50 to 870 μμg./ml. (with an average of about 200 to 300 μμg./ml.). It has been reported that the B_{12} content of blood (or serum) is lowered in certain anemias (especially pernicious anemia, confirming previous findings) (78, 86, 142, 143, 219 to 224). In fact, low serum B_{12} values may be evident in individuals before frank anemias are encountered (219, 220). Serum values are raised by oral or parenteral administration of B_{12} (86, 142, 143, 219 to 225) and also are raised in cases of allergic eczema (80), myeloid leukemia (140, 226), iron deficiency anemia (225), and in diabetes with retinopathy (227). They are unchanged or variable in lymphatic leukemia (140, 226), tropical anemia (223), diabetes without retinopathy (227), or in the serum of premature infants (228). Urinary excretion of B_{12} is raised by high doses of B_{12} (142, 143, 220, 221, 222, 224, 229, 230). Becker *et al.* (231) state that after test doses urinary B_{12} is low in uncontrolled diabetes and higher than normal in diabetes with retinopathy.

Pernicious anemia and other megaloblastic anemias.—Interest continues unabated in this subject and a complete coverage of all clinical aspects, especially case histories, is beyond the scope of this review. Most of the basic studies have been concerned with absorption of B_{12} in patients with pernicious anemia (see intrinsic factor). B_{12} continues to be used with apparently complete success in treatment of pernicious anemia (232, 233, 234 and later references) in spite of the caution of Murphy (235) who stated that "further critical study is necessary before vitamin B_{12} is accepted as a complete substitute for either whole liver or liver extracts."

Meyer *et al.* (236) found that as little as 0.5 to 1 μg. B_{12}/day by intravenous route was effective in treatment of pernicious anemia. B_{12b} may be less effective than B_{12} (237) while certain naturally occurring B_{12} polypeptides of unknown structure in liver appear equal to B_{12} (238, 239). Studies have continued on nasal or respiratory tract inhalation therapy of B_{12} for treatment of pernicious anemia with favorable results over long periods of time (230, 240). Such methods appear as effective as injected routes and are simple and safe. It is interesting that IF^1 activity does not appear necessary for B_{12} absorption in the respiratory tract (230).

Also of interest is the fact that satisfactory clinical and hematological remissions in pernicious anemia can be obtained with oral B_{12} without IF if initial doses of as much as 2 to 9 mg./day are given followed by smaller daily doses (142, 143, 222, 241, 242). Toxic reactions have not been seen with

these high doses except in one report of a questionable nature which described three cases of an allergic type reaction (243). Commercial preparations of B_{12} are now available combined with IF. Such preparations, containing relatively small amounts of B_{12} and only 0.05 to 0.30 gm. of IF, have proven very effective when given daily by the oral route (244 to 248).

Several studies have appeared on the use of B_{12} and FA[1] in megaloblastic anemias [see review by Davis & Brown (249)]. In megaloblastic anemia of pregnancy hematological responses to B_{12} are suboptimal and inferior to results obtained with FA alone (250, 251). A relationship between intestinal flora and the occurrence of megaloblastic anemia in Africans has been studied by Foy & Kondi (252, 253) who reported that penicillin given orally or by injection causes a hematological response in many individuals. Their data suggest that penicillin influences the biosynthesis and utilization of intestinal B_{12} with the types of diets consumed (high carbohydrate and low protein). The presence of parasitic worms in the intestine may also cause megaloblastic anemia which responds to B_{12} supplementation (253, 254).

A synthetic compound of undisclosed origin, said to be a member of the B_{12} group and with an empirical formula of $C_{54}H_{66}O_{12}N_{14}PCo$, was stated to be successful in treating 17 out of 20 patients with various types of anemias (255). More details are necessary before this work can be accepted.

Intrinsic factor and B_{12} binding proteins.—Reports of chemical studies on concentrated forms of IF, combined with B_{12} in varying degrees, have appeared this past year (246, 256, 257, 258). Also, excellent reviews by Castle (234) and others are available (1, 5, 259, 260).

The isolation of what appears to be pure IF when tested by accepted criteria of protein purity has been announced by Latner *et al.* (261, 262, 263), although full details are not yet available. The resulting product, obtained from hog gastric mucosa, is a mucoprotein with a molecular weight of about 15,000. As little as 1 mg. of material per day gave a response in humans as measured by a technique involving oral administration of the mucoprotein with a dose of radioactive B_{12}. [Variations of this method of measurement of IF activity or B_{12} absorption have been studied further this past year, in which the B_{12} excretion, blood level, or radioisotope emission over the liver is measured, following the administration of a dose of radioactive B_{12}. The methods appear very successful and shorten considerably the time necessary for IF assay (260, 264 to 270)]. The IF preparation of Latner *et al.* (261, 262, 263) contained 10 per cent nitrogen, 6.5 per cent hexosamine, 2.2 per cent fucose (deoxygalactose), 13 per cent reducing sugar expressed as glucose, 1.04 per cent tryptophan, and 1.96 per cent tyrosine. Glass (271) has criticized the reports of Latner on several grounds, but it would appear that satisfactory evidence is now available that an IF (perhaps there are more) has now been isolated. Recently, Williams *et al.* (272) obtained a highly potent IF preparation with a molecular weight of about 5,000 and containing 15.2 per cent glucosamine and 11.8 per cent nitrogen. It was effective in patients with pernicious anemia at a daily dose of 1 or 2 mg. It appears to be distinct from Latner's preparation.

Additional studies on the site of IF secretion in the body have been made. After gastrectomy in patients little or no IF is excreted (267, 269, 273, 274), confirming previous studies. The source of IF in the pig may be different from that in human. Careful measurements of activity have confirmed that both the pyloric mucosal (275, 276, 277) and duodenal secretions (275) of the pig have IF activity. Little or no activity has been found in dried fundus or uterus muscularis of the pig (277, 278).

Important studies by Wijmenga *et al.* (279, 280) have resulted in the isolation from hog gastric mucosa of several highly purified proteins with high binding capacity for B_{12}, but with different properties than IF. One of the proteins is pink as a result of the presence of B_{12} (as high as 18.5 μg./mg.). It has a molecular weight of about 70,000 and appears to be a mucoprotein. The cyano-group is still present in the molecule. The substance appears to be identical with the "B_{12}-binding factor" of hog mucosal extract but does not have IF activity in a clinical test (although partially purified preparations did have activity). Thus, there is now direct conclusive evidence that IF activity is not correlated with B_{12}-binding capacity, a fact strongly suspected previously (1, 234, 256).

Other sources of B_{12}-binding substances besides the stomach exist in nature. The isolation of a B_{12} protein complex from sow's milk (as well as pig stomach) has been described by Gregory & Holdsworth (281). The substance is a pink protein which appears similar to the protein described by Wijmenga *et al.* (279, 280). The total nitrogen is 16.1 per cent and it contains 23.6 μg. of B_{12} per mg. with a suggested molecular weight of about 55,000. It appears to have an amino acid pattern typical of proteins, but it has an unusually high tyrosine content (17.6 per cent). Since it contains 7 per cent of hexosamine, the authors suggest that it is a glycoprotein rather than a mucoprotein (281). The substance is also present in milk of woman, rat, goat, and cow but to a lesser extent. The B_{12} portion is largely unavailable to *L. leichmannii* unless digested with trypsin or papain and heated with the assay medium.

What appear to be other B_{12}-binding substances with various properties have been studied in crude form in egg yolk (282), in liver extracts (283), in gastric juice (284, 285), in feces of normal and anemic persons (167), in hog mucosal extracts (286), and in blood serum (285, 287, 288, 289). It has been reported (287, 288) that α-globulin fractions in the blood are chiefly responsible for its binding activity and suggestions have been made that the bound substance may have a physiological role in the transportation of B_{12} in the blood stream. The point of attachment for one of these binding substances appeared to be the cobalt and the 5,6-dimethylbenzimidazole part of the B_{12} molecule (286). Although there are similarities between some of these substances, undoubtedly at least several B_{12}-binding proteins or polypeptides exist in nature. It is of interest that pseudo B_{12}, Factor A, and Factor B also combine with the above protein from sow's milk and hog stomach in the same manner as B_{12} (290).

Absorption of B_{12}.—The mechanism by which B_{12} is absorbed through

the wall of the intestine is not understood (see page 352 for references to techniques of measurement). Glass *et al.* (291, 292) have suggested that an "intramural 'intestinal B_{12}-acceptor' exists, which may be responsible for the partial mucosal block" to B_{12} absorption in humans analogous to the role of apoferritin. This could conceivably explain the poor absorption of B_{12} in pernicious anemia and sprue, and the sharp decline in percentage absorbed when high doses are given to normal individuals (291, 292, 293). More proof is needed, however, for the existence of such a factor.

Other therapeutic uses of B_{12}.—If all the miraculous results reported in the world's literature on the beneficial effects of B_{12} on a variety of diseases (other than pernicious anemia) were true, B_{12} would certainly be one of the chief wonder drugs of the century! As with most new drugs many claims have been made for B_{12} which obviously will not stand the test of time. Fortunately, at least a few of the reports appear to be well-founded, and at present B_{12} is being used routinely in several diseases with varying degrees of success.

One recent development is the stated therapeutic success obtained in certain neurological diseases by the use of very large doses of B_{12} (up to 1 or 2 mg./day, given intramuscularly). This amount is considerably greater (approximately 20- to 100-fold) than the normal daily intake, and any benefits obtained should probably be considered attributable to a pharmacological effect rather than a vitamin action. B_{12} was reported to be successful in temporary treatment of trigeminal neuralgia (294 to 297) and has stated effectiveness in glossopharyngeal neuralgia (294), funicular myelosis (296), alcoholic polyneuropathy (298), sub-acute combined spinal cord degeneration without the usual anemia (273, 299), and in certain other types of nervous disorders, many of which require confirmation (300, 301, 302). The successful use of high doses of B_{12} in the treatment of diabetic neuropathy (303 to 306) was partially confirmed, but good contrary evidence is also available (307, 308, 309). It is possible, but not proven, that B_{12} may be effective in certain neurological diseases because of its influence on the nucleoprotein and lipide patterns of the myelin sheath of nerves as shown in studies with rats (310).

High doses of B_{12} have been reported to be successful in many other diseases, but many of the reports appear doubtful. They are cited here only briefly to show the scope of the diseases studied. All of these reports need confirmation with well controlled experiments. Reports have been made of inconsistently successful responses attributable to B_{12} in the treatment of cases of lupus erythematosus (311 to 314) and in certain joint diseases including bursitis (315, 316), cervico-brachialitis (317), and arthritis (316, 318). Single questionable reports, often based on very weak data, have stated that B_{12} has been successfully used for treatment of leukoplakia (313), seborrheic dermatitis (314), toxic basophile stippling (319), corneal lesions (320), some forms of anosmia (loss of sense of smell) (321), dyspepsia in infants (322), anemia of surgery (323), anemia from massive resection of the small intestine (324), lead poisoning in man (325), relief of pain from old

surgical lesions (326), herpes zoster (327), and even of hemophilia (328) and neuroblastoma (329).

B_{12} has been found inactive in treatment of alkaptonuria (330), osteoarthritis (331), pulmonary tuberculosis (332), hemophilia (333, contrary to 328), multiple sclerosis (296), muscular dystrophy (296), and of questionable use in hemolytic anemia (334). It is no doubt more than a coincidence that the experiments reporting negative results usually are better controlled than the many questionable reports showing a positive effect in a variety of diseases.

B_{12} in Animal Nutrition

Interest continues unabated in B_{12} in animal nutrition and metabolism. Most reports have dealt with interrelationships of B_{12} to other biologically active compounds. Fundamental animal studies not previously reviewed are included in this section.

Antibiotic relationships.—It is well known that antibiotics may have a sparing action on the requirement for many of the B vitamins including B_{12} [see review by Stokstad (335)]. Confirmatory studies with new techniques have shown a sparing action of antibiotics on B_{12} in the rat (151, 336), pig (337), chick (165, 338), and sheep (339). In most of these studies the feeding of the antibiotic was associated with marked changes in the intestinal flora and in B_{12} synthesis and storage. It appears, therefore, that the sparing action of antibiotics on the B_{12} requirement for growth is chiefly indirect and nonspecific. In evidence for this conclusion, a number of recent papers show no direct antibiotic and B_{12} relationship (153, 154, 340 to 344).

Hormone relationships.—Again, no direct metabolic relationship has been demonstrated between B_{12} and any hormone. However, several interesting indirect relationships were reported which show that certain hormones will either increase the requirement for B_{12} or that large amounts of B_{12} will overcome their toxic effects. Studies of this nature were made with testosterone and cortisone in man and rabbits (231, 345); cortisone (346), ACTH (347), and thyroxine (149) in rats; and thyroprotein in mice (348). In studies with the rabbit Lederer & Duckers (349) reported that B_{12} is not capable of inhibiting the diabetogenic action of cortisone. Future studies are needed to more fully understand possible hormone-B_{12} relationships.

Rat.—Histological studies of B_{12}-deficient rats fed diets containing iodinated casein showed a marked change in the testis and the thyroid but not in the liver, muscle, or bone marrow (350). The testis showed decreased spermatogenetic activity, shrinking of the seminiferous tubules, and degeneration of interstitial tissues, all of which were counteracted by dietary B_{12}. Histological changes were also seen in nerve tissue of B_{12}-deficient rats and were described in detail by Alexander (310). Other reported symptoms of B_{12} deficiency included retardation of sexual maturity of females (351); faulty reproduction (352); a small increase in basal metabolism (353); decreased resistance to cold stress (354); congenital malformations, including

hydrocephalus and eye and bone defects (355); and the occurrence of fatty livers or kidney lesions (only seen when a carefully adjusted diet also low in choline, methionine, and protein is used) (198 to 200, 356, 357). Perdue & Phillips (358) could not demonstrate a requirement for B_{12} in progeny of rats fed an all-plant diet. Apparently, conditions for this demonstration are somewhat sensitive.

Additional studies were made on the effect of various changes in the protein (341, 351) or carbohydrate (151, 351) content of the diet on the B_{12} requirement of the rat. The various effects observed appear unrelated and may be a result of the influence of the dietary components on intestinal synthesis or to their content of B_{12} or B_{12}-sparing factors, such as methionine (5, 359). Improved biological efficiency of dietary protein attributable to B_{12} feeding was reported in studies with rice (360), raw soybean (361, 362), casein (362), and with supplemented wheat proteins (363).

A number of miscellaneous studies were made with B_{12} in the rat including studies on the B_{12}-sparing action of 1-L-arabinofuranosyl-5,6-dimethylbenzimidazole (10), cobalt (364), and a synthetic cobalt-nucleic acid complex (91); on the protective effect of B_{12} on the toxicity of emetine (365), allyl formate (366), and molybdenum (367); on a possible thiamine (150, 368) and riboflavin (150) interrelationship; on a sex difference in response to B_{12} when added to a deficient ration (response seen in males only) (369); and on the unessential role of IF in absorption of B_{12} in deficient rats (370). Most of these studies require confirmation or publication of further details.

Mouse.—In addition to the studies mentioned previously with the mouse (116, 342, 348), Lee *et al.* (371) reported a possible relationship between α-tocopherol and B_{12} in partially overcoming reproductive failure in female mice fed a synthetic type diet; however, the evidence offered is not very convincing. Woolley (117) reported a partial and temporary regression of mammary tumors in mice by the administration of three different antimetabolites of 1,2-dimethyl-4,5-diaminobenzene, a suggested B_{12} precursor.

Pig.—Several improved diets for the study of B_{12} deficiency in the young pig have been suggested. Firth *et al.* (201) used a diet for the baby pig consisting of soybean protein (29.4 per cent), lard (30.5 per cent), and glucose (30.5 per cent) supplemented with methionine, minerals, and vitamins (except choline). A marked B_{12} deficiency was observed within five weeks, resulting in refusal of food, vomiting, weakness, death, and low B_{12} content of the liver. Neither fatty infiltration of the liver nor renal damage was observed. A similar study by Bauriedel *et al.* (155) made use of 1 to 2 day-old unsuckled pigs fed a "synthetic milk" up to 13 days of age, followed by a solid soybean protein ration up to 8 weeks. A B_{12} deficiency resulted in reduced growth and lowered amounts of B_{12} in the blood, liver, kidney, pancreas, and feces.

Pigs fed corn-soybean meal diets required the same amount of B_{12} regardless of the level of protein (372) and in spite of previous indications of an interrelationship. Several other studies with practical type rations have

confirmed the importance of B_{12} in swine nutrition (373, 374). In fact, Henson *et al.* (375), obtained a growth response by adding B_{12} to rations containing 10 to 17 per cent of tankage, a meat by-product usually considered adequate in B_{12}. Other studies with the pig have been cited previously (153, 154, 337).

Fowl.—Some of the continued interest in B_{12} in fowl nutrition is attributable to the fact that the commercial availability of low-cost B_{12} concentrates has resulted in the addition of B_{12} to nearly all poultry feeds manufactured in this country, with a resulting saving in animal proteins. Further studies on the beneficial effect of B_{12} in various practical type rations for chicks and broilers (343, 376), for laying and breeding hens (161, 338, 343, 377, 378, 379), and for turkeys (344) have been published. Studies on the level of B_{12} required in the hen's diet under a variety of conditions show that a range of from 1 to as high as 10 μg. per kg. of diet may be needed for normal hatchability of eggs and growth of progeny (343, 378, 379). The Committee on Animal Nutrition of the National Research Council recently published tentative recommendations for minimum B_{12} requirements per kg. of diet as follows: growing chicks—9 μg., and breeding hens—4.4 μg. (380).

Ferguson & Couch (381) published further information on gross and histological anomalies of the B_{12}-deficient chick embryo. At least 25 per cent of 17 day embryos were found to have an enlarged thyroid, a thin-walled digestive tract, poorly developed feathers, edema, small and tendinous muscles, fatty livers, pale or dilated heart, and fatty kidneys. All anomalies were prevented by giving B_{12} either to the hen or injecting it into the egg prior to incubation. Few abnormalites of this nature have been reported, as yet, in B_{12}-deficient chicks or hens.

It is known that the growth of a B_{12}-deficient fowl is stimulated by compounds other than B_{12}. An occasional B_{12}-sparing effect of antibiotics (*loc. cit.*), of methionine (64, 377, 382), and of certain levels of cobalt (165, 166, 364) were reported, confirming earlier studies. Several studies were directed toward the development of conditions of dietary stress to produce a more severe B_{12} deficiency in the chick. For this purpose various workers used high soybean meal diets (383, 384), iodinated casein (162, 384), and high levels of vegetable proteins or fat (63). The use of 20 per cent fat appears to be most successful in the production of a B_{12} deficiency in the chick, suggesting a possible role of B_{12} in fat metabolism for this animal. The quality of soybean meal used in deficient diets was reported to affect the B_{12} requirements; underheated meals depressed growth in the absence of B_{12} (385); however, the experiment was poorly controlled. Other studies, requiring confirmation, have shown that dietary B_{12} may be necessary for the development of selenium toxicity under certain conditions (386); that B_{12} may reduce the growth of *Ascaridia galli* (roundworms) in chickens (387) (contrary to the results of Brody who used a deficient type of diet, 388); and that B_{12} may prevent cod-liver oil toxicity (389).

Ruminants.—After the rumen begins to function a normal ruminating animal obtains all the B_{12} it requires, and more, from bacterial synthesis. A number of factors are known to influence the amount of synthesis of B_{12} in the rumen. These include the type of ration (164), the amount or source of energy, sulfur, and methionine [in studies with an artificial rumen (390)], and, obviously, the presence of cobalt (139, 172, 391). The B_{12} synthesized in the rumen of the lamb may be very inefficiently absorbed into the body according to Kercher & Smith (392) but more evidence is needed. Stitt *et al.* (393) reported no consistent benefit in adding B_{12} to B_{12}-deficient rations for lambs, which again demonstrates the importance of rumen synthesis of this vitamin.

In the very young calf, however, a dietary source of B_{12} is required. Approximately 20 to 40 μg. per kg. of diet is needed for prevention of growth retardation, lack of appetite, muscular weakness, and a "white spotted kidney condition" [Lassiter *et al.* (394)].

Miscellaneous studies.—Several B_{12} studies were made with the rabbit, a species for which there is no dietary requirement known. In fact, according to Kulwich *et al.* (163), the amount of B_{12} recovered in the feces of rabbits fed a purified B_{12}-low diet was 221-fold greater than the intake. These workers also reported that coprophagy by rabbits on stock diets provided 42 per cent more B_{12} than would be available otherwise, and that the soft feces (voided at night) were 2 to 3 times as rich in B_{12} as hard feces (voided during the day). Other studies with the rabbit indicated that B_{12} may counteract lead-induced anemia (66, 395); protect against hepatic injury caused by CCl_4 intoxication (396); stimulate proliferation of the epithelium of corneal incisions (397); and may help to protect against cortisone-induced renal lesions (345).

Although the guinea pig requires a dietary source of most of the B vitamins, no requirement for B_{12} has been demonstrated yet [Reid (398)]. In a study with dogs, Rosenthal & Hampton (399) showed that B_{12} is absorbed primarily in the duodenum and not in the stomach. Lastly, it is of interest that excess dietary B_{12} was found to double the number of tumors in *Drosophila melanogaster* (400). The significance of this finding, if any, to higher animals is not known.

THE FOLIC ACID GROUP OF COMPOUNDS

(Pteroylglutamic Acid, Folinic Acid or Citrovorum Factor, and Related Compounds)

During the past year, a great many papers on the folic acid group of compounds [hereafter referred to collectively as folic acid (FA)] have appeared. There has been particular interest in the identification of the coenzyme form or forms of the vitamin, in the elucidation of the various steps involved in the transformation and especially the transfer of one-carbon units, and in the development of antagonists and their experimental and clinical application. Progress has been steady rather than spectacular.

Chemistry and Biochemistry

Although the chemistry of many different forms of FA has been thoroughly studied, it is not as yet entirely clear which form or forms occur in cells and tissues and serve as coenzymes in enzymatic processes. For a time it was generally believed that the citrovorum factor (CF), N^5-formyltetrahydro PGA, served invariably as the coenzyme, but in the past few years and particularly during the past year a great deal of evidence has been presented against this concept. In addition to CF, forms of FA which have been suggested as serving as a coenzyme in certain enzymatic processes have included N^{10}-formyl-PGA, tetrahydro-PGA and N^5-hydroxymethyltetrahydro-PGA as well as a FA compound of unknown structure, as discussed in the following sections.

Purine and pyrimidine synthesis.—Buchanan and co-workers (401 to 404) have described an enzyme system from pigeon liver which incorporates radioactive formate into the 2-position of inosinic acid. The reverse reaction, the formation of 4-(5)-amino-5-(4)-imidazolecarboxamide ribotide from inosinic acid in the presence of glycine, was also shown to be catalyzed by this enzyme system. Cu^{++} also was shown to be involved. The authors concluded that CF acts as a transformylating coenzyme in these reactions and postulated that glycine exerts its effect by acting as a formyl acceptor.

Greenberg (405) has summarized present concepts of the synthesis of purine nucleotide in pigeon liver (see also 406 to 410). Ribose-5-phosphate is converted to another ribose-phosphate derivative (the nature of which is somewhat in question) (401, 411, 412), then interacts with glycine and glutamine to form glycine-N-amido-5′-phosphoribotide, (NH_2CH_2CO-NH-ribose-5′PO_3H_2). This compound accepts an activated formyl group, ring closure occurs and ammonia is added enzymatically. The resulting aminoimidazole compound is carboxylated forming the well known 4-(5)-amino-5-(4)-imidazolecarboxamide-5′-phosphoribotide (1RMP5) which accepts an active formyl group with closure of the purine ring to form inosinic acid. Greenberg (405) also presented additional evidence to that previously reported to support the concept that the compound which first accumulated in sulfonamide-inhibited *E. coli* cultures is the ribotide, 1RMP5, rather than free aminoimidazolecarboxamide and that it is then degraded to the riboside and the free base. The imidazole-riboside (1R), it was shown, can be converted by typical ATP-kinase reactions to its 5′-phosphoribotide (1RMP5).

Greenberg's studies on the transformylation co-factor (405, 408) suggest that the co-factor is not CF but instead that it is a related compound of unidentified structure and that it can be derived from CF by activation with ATP.[1] This transformylation co-factor is extremely labile at neutrality, being converted during isolation to N^{10}-formyl PGA. It has properties similar to those of anhydro CF.

Silverman & Keresztesy, from their studies of conversions of FA compounds from one form to another by liver homogenates, had reported previously on a product which was intermediate in the conversion of the "bound-

liver" form (presumably the coenzyme bound to liver protein) to CF (413). The form finally isolated (414) was N^{10}-formyl PGA. The isolated compound, however, was not converted to CF by liver autolysates, and the authors concluded that a labile intermediate, which could be converted to CF by their liver preparation, had been stabilized or degraded to N^{10}-formyl PGA. They showed also that N^{10}-formyl PGA, PGA, or CF can be inactivated by incubation with fresh rat liver preparations.

Goldthwait & Peabody (415) and Goldthwait (416) presented evidence that the incorporation of formate into the 2 and 8 positions of purines is via identical or similar pathways. Note is made that under the conditions of their experiments the co-factor preparation used can be replaced by CF. Strength *et al.* (417) showed that injections of aminopterin reduced the DPN^1 content of rat livers and that incubation of rat liver homogenates with the antagonist decreased both the endogenous respiration and the DPN content. ATP counteracted the effects observed with the homogenates.

Weaver & Shive (418) have shown that when the utilization of 4-(5)-amino-5-(4)-imidazolecarboxamide is inhibited by low concentrations of aminopterin, this inhibition can be overcome by thymidine but not by folinic acid-SF (CF) alone. They concluded from their data that thymidine plays a catalytic role in the utilization of the amine. Sauberlich (419) has reported studies on a variety of organisms. He concluded that CF was involved particularly in thymine and thymidine formation and that neither homocysteine nor 4-(5)-amino-5-(4)-imidazolecarboxamide appeared to be involved in the actions of CF. Elwyn & Sprinson (420) from isotope studies in rats found that the hydroxymethyl group of serine is the major source of the methyl group of thymine. Their results suggest, also, that there are separate and distinct mechanisms for the incorporation of atoms 2 and 8 of purines.

Berg (421) from his studies of the incorporation of formate-C^{14} into either methionine, the β-carbon of serine, or the ureide carbons of purines, proposed the hypothesis that an intermediate formed from formate and homocysteine is a common precursor in these syntheses.

Serine metabolism.—There has been considerable study during the past year of glycine-serine interconversions both by microorganisms and by tissue extracts, and evidence has been presented that the active co-factor is not CF. Differing suggestions have been made as to its identity.

Rauen & Jaenicke (422) have reported evidence for serine synthesis from N^{10}-formyl-PGA and glycine in tissue homogenates and extracts.

Blakley has presented new data (423, 424) showing that formaldehyde but not formate can be added to glycine to form serine, the necessary enzyme activity being contributed by soluble preparations from pigeon liver. The addition of ascorbic acid and anaerobic conditions favored the reaction. When the enzyme preparations were well dialyzed they could be reactivated by ATP, DPN, and PGA. The most active form of the vitamin for this purpose, however, was tetrahydro-PGA. The interconversion of glycine and serine was inhibited by 4-amino-PGA in the presence of PGA or the N^{10}-formyl derivative but only slightly in the presence of CF or tetrahydro-

PGA. Blakley concluded that before activating the serine-glycine interconversion PGA undergoes enzymic reduction to the tetrahydro form which plays a central part in the transfer of one-carbon units, probably by combining reversibly with formaldehyde with formation of an imidazolidine derivative.

Somewhat similar results were presented by Kisliuk & Sakami (425, 426). It was also noted by these investigators that homocysteine increased 4-fold the glycine-C^{14} incorporated into serine by their pigeon liver extracts. Activity was restored to inactivated preparations by addition of tetrahydro-PGA alone but not by PGA or CF. The authors presented the hypothesis that hydroxymethyltetrahydro-PGA or a derivative is the one-carbon unit directly involved in serine biosynthesis.

Elwyn & Sprinson (427) extended the earlier work of Elwyn *et al.* (428) on the stability of the carbon-hydrogen bond in transfers of the β-carbon of serine. Their recent studies (427) were on the interconversion of serine and glycine and again the β-$C^{14}\beta$-D ratio was unaltered.

Lascelles *et al.* (429) described the conditions necessary for the growth of *Leuconostoc mensenteroides* P60 in the absence of serine and showed that in the absence of excess CO_2, pyridoxal, CF, and high concentrations of glycine are needed. PGA was inactive. Lascelles & Woods (430) have studied the formation of serine from glycine and formate by *Streptococcus faecalis* R. They concluded that before it is active in serine synthesis by this organism, PGA is transformed to a reduced and formylated form of FA similar to but probably not identical with CF. They note that their results are compatible with the postulations of Blakely (423, 424) or of Kisliuk & Sakami (425, 426).

Histidine degradation.—Rats deficient in FA have been shown to excrete large amounts of a derivative of glutamic acid in their urine (431), and the amount excreted is greatly increased by the administration of histidine (432). Studies with labeled histidine showed that the glutamic acid derivative arises from histidine catabolism (433). Studies on the degradation of histidine by enzyme preparations resulted in the formation of an identical compound (434) which was identified as α-formamidinoglutaric acid (435). Its synthesis has been described (434, 436). Because of the known participation of FA coenzymes in one-carbon unit transfers, it is tempting to conclude that this is the normal pathway of histidine catabolism which is completely or almost completely blocked in FA-deficient animals. However since the addition to the diet of the deficient animals of histidine (or tryptophan) with ascorbic acid and B_{12} permits growth and partially corrects the blood dyscrasias (437), the situation may be more complicated than first appears. Also, Silverman *et al.* (438) showed that after subcutaneous injection into normal rats, the urinary compound α-formamidinoglutaric acid, is excreted unchanged. Silverman *et al.* (439) have recently shown that, when the deficient diet is supplemented somewhat as above, a material with the growth properties of isoglutamine is excreted in the urine.

Cell division.—Jacobson (440) reported additional experiments from

which he concludes that CF is essential for cell division and, more specifically, for the step from metaphase to anaphase, i.e., for the parting of the chromosome halves. This step, it was shown, could be prevented by aminopterin or A-methopterin in bone marrow preparations (from acute human and mouse leukemia) in intestinal epithelium of the mouse and in chick embryo cells grown *in vitro*. PGA did not overcome this effect when tested directly on dividing cells grown *in vitro* even when applied 1 hr. before the antagonist. However, CF when applied simultaneously with aminopterin did prevent the arrest in the metaphase stage.

Other metabolic processes.—Friedmann *et al.* (441) showed that formate oxidation is impaired in FA-deficient rat tissues. Catalase activity in liver, but not in heart or kidney, was lowered indicating that catalase may not always be the limiting factor in formate oxidation. Weinhouse & Friedmann (442) presented evidence to indicate that FA-deficient rats had an increased rate of formate production from the methyl groups of methionine, choline and sarcosine (but a lower rate from the α-carbon of glycine). They suggested that the excess production of free formic acid is attributable to normal pathways of utilization being blocked by the lack of a transferring agent.

Rege & Sreenivasan (443) showed that chlortetracycline (aureomycin) inhibits the production of both pentosenucleic acid (RNA) and deoxypentosenucleic acid (DNA) by *L. casei* and that FA (and to a lesser extent B_{12}) protects against this action. Martin & Totter (444) have confirmed earlier workers in the observation that formate incorporation into intestinal RNA[1] is impaired in rats treated with aminopterin. They have shown, however, that under their experimental conditions, liver RNA showed a markedly increased specific activity. Dinning *et al.* (445) reported that bone marrow cells from FA-deficient chicks showed a complete loss of choline oxidase activity but an increase in some other processes which Dinning & Day (446) have noted includes incorporation of formate into nucleic acids.

Other effects resulting from FA deficiency includes the report by Brown (447) who found an adverse effect of the deficiency on nitrogen retention in chicks. Also, Vorhaus & Vorhaus (448) reported a slight beneficial effect of an excess of FA on CCl_4-induced hepatic injury in rats. Further work has been reported on endogenous bacterial infections in FA-depleted rats by Asenjo (449) and on malformations in offspring from FA-deficient pregnant rats by Monie *et al.* (450) and by Sansone & Zunin (451). The role of FA in hormonally-induced tissue growth has been further studied by Silver (452) and by Brown (453, 454), the latter reporting beneficial effects from dietary penicillin (453) and deoxypentosenucleic acid administration (454) in overcoming the impaired oviduct response to estrogen in the FA-deficient chick. Schwartz *et al.* (455) have extended their work on the biosynthesis of ascorbic acid in the rat and concluded that a deficiency of FA inhibits this process. Kirsanova *et al.* (456) reported that FA-deficient chicks have an elevated level of ascorbic acid in the spleen, probably reflecting an increased functional activity of this organ. Giroud *et al.* (457) reported further evi-

dence that FA affects pantothenic acid levels, and Zumoff (458) noted a high blood level of uric acid in a case of FA deficiency.

CF from PGA.—Further studies on the metabolic synthesis of CF from PGA have been reported by Mitbander & Sreenivasan (459), Doctor and co-workers (203, 460 to 463), Nichol (464), Zakrzewski & Nichol (465), by Bond (466), and by Foley & Haley (467).

Antagonists

Several new A antagonists have appeared. Fairburn *et al.* (468) described the synthesis of four oxygen analogues of pteroic acid and PGA in which the N^{10} nitrogen is replaced by oxygen and have shown that they are FA antagonists. Angier *et al.* (469) synthesized a number of pteroic acid analogues containing arsenic and stated that several showed moderate oncolytic activity. New pteridyl-sulfanilamides with activity against *Streptococcus lactis* but not against Walker rat carcinoma were described by Fahrenbach *et al.* (470). Spickett & Timmis (471) and Felton *et al.* (472) described other new compounds showing weak anti-FA activity.

Studies with bacteria.—Hakale and co-workers (473) and Foley & Watson (474) studied the development of resistance to FA antagonists by *S. faecalis*, and Prusoff *et al.* (475) have reported on the effect of 6-azathymine on several microorganisms. Nichol *et al.* (476) and Jackson and co-workers (477) showed that apparent utilization by microorganisms of four amino analogues and of chlortetracycline, respectively, was attributable to contaminants. Dewey & Kidder (478) presented a similar explanation for earlier results with a variety of FA antagonists on *Tetrahymena pyriformis* and have studied other compounds which are inhibitory to this organism. Elion *et al.* (479) studied particularly the synergism which sometimes occurs with combinations of antimetabolites. Greenberg (480) extended his earlier studies of antimalarials which are also FA antagonists by showing that the antimalarial effect of sulfadiazine can be reversed by FA and various derivatives. Collier & Phillips (481) employing *L. citrovorum* showed that a number of 2,4-diaminopteridines are CF antagonists. Nimmo-Smith & Brown (482) made similar studies using *S. faecalis* R.

Studies with animals.—Nadel & Greenberg (483) showed that A-methopterin and a diaminopyrimidine have a synergistic action in the treatment of leukemic mice. Asano (484) described the inhibitory effect of aminopterin on the growth of Yoshida sarcoma, and Nichol (485) studied the mechanism of resistance to FA antagonists by leukemic mouse cells. Altland & Highman (486) showed that the polycythemia produced in rats by altitude exposures could be prevented by 9-methyl-PGA but that the altitude exposures did little to lengthen the survival times of rats fed the antagonist. Skipper *et al.* (487) showed that cortisone reduces the antileukemic activity in mice of A-methopterin. Barnard & Freeman (488) reported that certain water-soluble cupri-chlorophyllins protect weanling rats from aminopterin toxicity. This requires confirmation. Goldin and associates (489, 490, 491) studied

the effect of concomitant administration of CF with aminopterin in leukemic mice and have shown that best results were obtained by administering the CF 12 to 24 hr. after the aminopterin. Lewis *et al.* (492, 493, 494) showed that female rats are more sensitive to aminopterin than males and that adrenalectomy both increases the sensitivity and causes the sex difference to disappear.

Clinical studies.—The antimetabolites are widely used in the treatment of leukemia. They do not cure the disease but do cause remissions and, particularly in acute leukemias, increase the life span. Several clinical reports have appeared within the past year. Burchenal (495, 496) made a general report on the clinical management of leukemias and discussed the rationale of chemotherapy (495) as well as the effects of a number of the newer antimetabolites (496). Waisman & Harvey (497) presented evidence that antagonists arrest the leukemic infiltration of bone and permit, during periods of remission, growth of bones at a normal rate. Holthaus (498) reported on the chemotherapy of the lymphomas and leukemia. Murphy *et al.* (499) described clinical trials with dichloro- and monochlorophenyl analogues of diaminopyrimidine and concluded that they are FA antagonists.

Assay

Assay methods for total FA and for individual members of the group are still not completely satisfactory. However, some useful techniques and improvements on older methods have appeared in recent months. Zakrzewski & Nichol (500) described a method for the chromatographic separation of PGA and related compounds and their estimation by fluorometric assay. This method has proved useful in showing the contamination of antagonists with PGA (476). Andreeva (501) studied further his fluorometric assay method and has used it for the determination of FA in grain, leguminous products, fruits, berries, vegetables, and animal tissues. Palonovski & Lévy (502) have described a microtechnique for CF determination. Brody (503) used the weight of the thymus gland of chicks infected with *Ascaridia galli* as a rough assay tool. Saxena *et al.* (504) used a chick growth assay method to study FA levels in natural feedstuffs. Ericson and co-workers (119, 120, 505, 506, 507) used some new assay procedures as well as standard microbiological techniques for the determination of FA in marine algae, lichens, and seaweeds.

Iwai and co-workers (508, 509, 510) determined FA in plant tissues, Ramachandran & Basu (511) in milk and milk products, Hall *et al.* (512) in fresh and dried figs, Teply *et al.* (513) in canned foods, Evans *et al.* (514) in fresh and stored eggs, and Toporek *et al.* (515) in tissues of FA-deficient rats.

Usdin (516) and Toennies *et al.* (517) studied the nature of the FA compounds in blood and Bleiler & Parsons (518) studied the excretion of FA compounds on a supplemented diet in studies with women.

Nutritional Studies

Animals.—Further studies have been made of FA deficiency in the dog, guinea pig, gosling, and monkey.

Afonsky (519) noted that a dog (a single animal) developed FA deficiency on a purified diet which was devoid of or very low in this vitamin. The deficiency syndrome, counteracted by PGA, was characterized by hypoplasia of the bone marrow, hypochromic anemia with a tendency to microcytosis and glossitis. Other dogs on the same diet showed no deficiency signs, a situation reminiscent of our experience with rats (520). Woodruff *et al.* (521) reported that FA deficiency in the immature guinea pig is characterized by growth failure, leucopenia, anemia, and immaturity of the erythroid precursors in the bone marrow. The deficiency syndrome was produced by the omission of PABA and PGA from a purified diet or by the inclusion of both PABA and sulfasuxidine. Reid (398) has continued her earlier work (522) on the essentiality of FA for the guinea pig and reports moderate anemia and leucopenia, aplasia of bone marrow, fatty infiltration of the liver, and hemorrhagic adrenals in the deficient animals. Briggs *et al.* (523) showed that a cervical paralysis occurs on goslings reared on a purified diet deficient in FA, which was cured by injections of PGA. Tappan & Elvehjem (524) have continued their studies with monkeys and have shown that generally adequate diets require supplementation with FA as a result of the high requirements of this species.

Microorganisms.—Hendlin (69) has reviewed recent work on the nutrition of microorganisms and studies have appeared by Kidder (525), Broquist (526), Havas & McGeady (527), Steinman *et al.* (528), Jandl (529), and Dewey & Kidder (530).

Humans.—Very little new has been reported concerning the use of FA or related compounds in the clinic. Tropical sprue was shown by Suárez *et al.* (531) to respond no better to CF than to PGA. In nutritional megaloblastic anemia, CF was reported by Watson *et al.* (532) to be comparable to PGA in its clinical usefulness. Jandl & Gabuzda (533) showed that the anemia of scurvy responds to PGA but that this effect of PGA is potentiated by the simultaneous administration of ascorbic acid. From a study of FA levels in the blood after test doses of PGA, Spray & Witts (534) concluded that their results lend support to the view that there may be an increased demand for FA and its derivatives in untreated pernicious anemia. Pernicious anemia patients under treatment with B_{12} and a patient with scurvy showed abnormally low levels of FA in the plasma and urine after a test dose while patients with leukemia showed fairly low levels. In hemolytic anemia, also, there seemed to be a slight indication for an increased demand for FA. Lawson & De Garis (535) reported that multigravid patients with a bad obstetrical background have shown improvememt when a FA deficiency is detected and corrected. Nieweg *et al.* (223) divided megaloblastic anemias into two groups, those in which neurologic disorders may occur

responding to B_{12}, the others to FA. They propose from their data, also, that B_{12} influences the storage or intake of FA and that the two vitamins act as coenzymes at different stages of nucleic acid synthesis.

Bacterial or Other Synthesis of FA

Many investigators are convinced that animals which do not require a dietary source of FA, such as the rat, obtain their necessary supply of this vitamin from intestinal synthesis by microorganisms. New evidence in favor of this concept has appeared within the past year. In addition, some evidence that synthesis of FA occurs in mammalian tissues (rat) will be reviewed.

Ford *et al.* (536) showed that the refected rat excreted substantial quantities of a number of vitamins, including FA, in the urine and that there were also appreciable quantities in the cecal material and feces. When the diet was cooked marked lowering of the amounts in cecal material and feces and some reduction of the amounts of most vitamins, including FA, in the urine was observed. When sulfonamides were included in the diet the amounts of all vitamins, especially FA, were lowered in the cecal material, and there were also reductions in the feces and urine.

Briggs *et al.* (537) showed that chlortetracycline in the diet may increase the severity of FA deficiency signs in the chick and interpret their results on the basis of changes in the environmental or intestinal microflora. Monson *et al.* (538) explained the increased growth in chicks brought about by supplementing the diet with antibiotics on the basis of the appearance of intestinal coliforms that produce increased amounts of FA. A later paper by Rhodes *et al.* (539) gives the results of a more complete study, and they suggest that the antibiotics may have acted not only by promoting greater synthesis of FA (and other growth fctors) by coliform bacteria but also by reducing the competition for FA or other essential compounds by the lactobacilli. Wichmann and co-workers (540) presented results from which they conclude that intestinal synthesis of FA takes place in the guinea pig and that on a suitable diet this synthesis is sufficient to meet the total FA requirements of the animal.

Some years ago Daft (541) presented results on the therapy of granulocytopenic rats with PGA or amino acids from which he concluded that PGA can be synthesized by animal tissues. During the past year Luckey & Reyniers (542) showed that germ-free rats, where, of course, bacterial synthesis is excluded, have several times the "output" of FA (amount in rat carcass at end plus amount in waste and excreta) as could be accounted for by the "input" (amount in rat carcass at start plus amount eaten in diet). They concluded that "tissue synthesis of vitamin B_{10} (FA and CF) is indicated."

CHOLINE

The entire subject of choline was reviewed in 1954 by Best and others (543, 544).

Assay and distribution.—An extremely sensitive chemical method for the estimation of as little as 5 μg. of choline in plasma was described by Appleton *et al.* (545). It is based on the determination of the ultraviolet absorption of choline periodide in ethylene dichloride. Modifications of standard chemical methods were made for choline estimation in vegetable materials (546, 547), in eggs (548), in serum of carcinoma patients (549), in bile (550), in urine (551) and for choline in pharamaceutical mixtures (552, 553, 554). The general subject of chemical estimation of choline was recently reviewed by Engel *et al.* (555).

Factors affecting the level of free choline in the plasma of the dog, rat, and man were studied by Bligh (556, 557). It was found that rapid disappearance of injected choline from the blood was attributable to choline oxidase activity of the liver and kidney. However, injected choline also may be rapidly excreted as determined in studies with man by Steigmann *et al.* (558). In the rat Bligh found that the plasma level of choline was reduced from a normal of 1.3 to 1.9 μg./ml. to a level of 0.6 to 1.1 μg. by a chronic deficiency of choline (557). Values for free choline in plasma of man obtained by Appleton *et al.* (545) were about twice as high (averaging about 4.4 μg./ml.) as those of Bligh, perhaps as a result of improved assay techniques.

Values also became available for the choline content of various materials including milk from several species (559), liver and urine of the pig (201), rat tissue and urine (560, 561), eggs (at various stages of incubation) (562), egg noodles (for determining egg content) (563), foods common to Bengal (564), and medicinal plants (565). An interesting new ester of choline, urocanylcholine, has been found in *Murex trunculus*, an invertebrate (566).

A method for the estimation of betaine in plant tissues was described by Cromwell & Rennie (567). They reported that the synthesis of betaine in the tissues of higher plants takes place largely as the result of oxidation of choline (568). The betaine formed accumulates in the stems and leaves, with values as high as 5 per cent of dry weight reported in the leaves of *Beta vulgaris* (sugar beet) (567). Several other leafy plants contained over 1 per cent of betaine. Since betaine has distinct choline sparing properties for most animals, the betaine content of foods should also be considered in assessing foods for total choline activity.

Ahmad & Karim (569) showed that various factors, including potassium, NH_3, and nitrate, affect the biosynthesis of choline in plants. Cromwell & Rennie (568) also described factors affecting choline biosynthesis.

Fatty livers.—In addition to the review by Best *et al.* (543), the subject of choline and its relationship to cirrhosis and fatty infiltration of the liver was reviewed by Schwarz and others (198, 199, 569a to 575). Current publications on this subject emphasize the complexity of the problem and the interrelationships of various nutrients, such as dietary choline, fat, FA, B_{12}, labile methyl groups, and amino acids, in the development of fatty livers. Of importance is the dietary level of protein and the balance of amino acids, especially threonine as demonstrated by Harper *et al.* (576). Liver

damage from protein or threonine deficiency, although related, may be differentiated by histological tests from fatty infiltration attributable to choline deficiency (357, 577, 578). The prevention of fatty livers in choline-deficient rats by heparin (579) and by proteolytic enzymes (580) has been indicated in experiments which require confirmation.

Casselman (581) reported that the formation of ceroid (a yellow lipide pigment) in livers of rats deficient in choline and vitamin E is influenced by autoxidation of unsaturated fatty acids in the tissues, and is partially inhibited by either α-tocopherol or methylene blue.

Although recent studies have given additional clues to the metabolic role of choline in preventing a rise of liver fat, it is not known with certainty if the lipotropic activity of choline is attributable to its methyl donor capacity, its incorporation into phospholipides, to an effect on fatty acid oxidation, or to some other mechanism, perhaps indirect. Still the predominant theory for the action of choline in reducing liver fat is the incorporation of choline into phospholipide. Along these lines, DiLuzio & Zilversmit (582) found that the addition of choline to liver slices from choline-deficient dogs caused a marked increase in the synthesis of choline-containing phospholipides but had no effect on synthesis of other phospholipides. That oxidation of fatty acids in the liver may be increased by choline, or an unknown derivative, was shown in studies with liver slices or homogenates from choline-deficient rats (583, 584). Further evidence for the role of choline in fatty acid oxidation in rat livers has been given (585, 586). Other reported effects of choline on animal livers which could conceivably play a direct or indirect role in liver-fat metabolism include an increase in hepatic circulation in dogs (587); a reduction of FA activity in liver autolysates from deficient rats (588); and effects on phosphorus metabolism (589) and on mitochondrial composition (590).

Although a rapid drop in liver-choline oxidase was reported in choline-deficient rats (591), the level of choline oxidase in normal livers from various animals (592) does not appear to correlate with the known susceptibility of these animals to fatty livers. Also, because of further evidence for the lack of specificity of choline oxidase (593), it is doubtful that this enzyme plays a direct role in the prevention or development of fatty livers. Bernhard *et al.* (594) suggested that the accumulation of fat in the liver of deficient rats was not attributable to increased fat synthesis but occurred as a consequence of histological damage.

In studies with the duck, Duchesne & Bernhard (595) noted that the total liver lipides varied with the dietary level of choline, casein, fat, and methionine, somewhat similar to findings with rats. Dietary cholesterol also caused an increase in liver fat. Additional studies giving further details on the known relationship between "cholesterol fatty livers" and dietary choline were reported by Ridout *et al.* (596, 597) and Raulin (598) in studies with the rat.

Cardiovascular disorders.—Several reports this past year from Best's

laboratory [Wilgram *et al.* (599, 600)] confirm the necessity of choline for maintenance of a normal cardiovascular system in rats. Twenty-two per cent of the animals fed choline-deficient diets containing natural fats had abnormalities of the cardiovascular system, whereas very few abnormalities were found in the controls. Examination of affected tissues showed aortic lesions consisting of intimal and medial deposits of lipide frequently associated with calcium salts. Lipide desposits were also found in coronary arteries and in myocardial muscle. Eventually, areas of frank necrosis developed in the muscle. As a result of these studies, Wilgram *et al.* (599, 600) suggested that the term "lipotropic" be extended to include the prevention of accumulation of abnormal fat in the heart and blood vessels in addition to the liver and kidney. This change in concept of the term "lipotropic" was recently questioned by Mann (601), who also pointed out that the diet of Wilgram *et al.* was low in methionine. Most of Mann's criticisms appear to have been satisfactorily answered by Wilgram *et al.* (602). Similar cardiovascular lesions in rats caused by feeding large doses of Vitamin D in choline-deficient diets were largely overcome by dietary choline (603). Demonstrating the complexity of this problem, Wissler *et al.* (604) reported that high choline intake (0.5 per cent) may increase the severity of atheromatous arterial lesions produced in rats by special dietary variation.

The exact role of choline in the prevention of cardiovascular lesions in rats is not understood. It may be related to the reported role of choline in the enhancement of fatty acid oxidation by liver, kidney, and heart tissues (583), or possibly, to the reported inability of the animal to form lecithin under certain conditions, as suggested by the studies of Pilgeram & Greenberg (605). No relationship between choline and atherosclerosis was found in studies of experimental cholesterol atherosclerosis in rabbits (606). Several conflicting reports have been made on the effect of choline or other lipotropic agents on arteriosclerosis in man (607 to 610). It must be concluded that for man no proven beneficial effect of choline has been seen, but further studies are needed. Because of the known suggested relationship between atherosclerosis and serum cholesterol, it is interesting that Ridout *et al.* (596) concluded from studies made with rats that "The same substances which prevent or cure fatty livers are also involved in the control of the cholesterol level in the serum." However, no support was found for previous claims of others that lipotropic substances reduce the concentration of bound cholesterol in serum.

Enzyme studies.—Several interesting reports giving new information on enzymes involved in the metabolism of choline were published this past year. Wittenberg & Kornberg (611) described an enzyme, choline phosphokinase, found in brewer's yeast which catalyzed the following reaction:

$$\text{Choline} + \text{ATP} \rightarrow \text{phosphorylcholine} + \text{ADP}$$

This enzyme, which also catalyzed the phosphorylation of ethanolamine and related compounds, was found in liver, brain, intestine, and kidney of several

species. It was suggested (611) that phosphorylated choline was the activated precursor of choline in lecithin; however, Kennedy (612) described an enzyme system in rat liver mitochondria which apparently incorporates free choline into the lecithin fraction by a pathway not involving phosphorylcholine.

Studies on a new spectrophotometric method for the determination of choline dehydrogenase in rat liver and kidney were described by Eichel [(613); see (543, 613, 614) for definition of enzyme systems]. Interesting studies by Rothschild *et al.* (614) show that the choline oxidase system is composed of the dehydrogenase, flavin-adenine-dinucleotide, cytochrome-*c*, and possibly Mg^{++}. The presence of —SH groups appeared necessary for choline dehydrogenase activity. Other studies with this enzyme showed that it was inhibited by ethylenimine structures (615); that FA may be necessary for enzyme activity in chick bone marrow (616); and that its activity is low in the livers of young rats but increases with age (617). According to Williams (618), ATP and DPN may not be necessary for the action of choline dehydrogenase.

Contrary to choline dehydrogenase, the level of betaine aldehyde dehydrogenase in rat liver is not affected by age (617). It was shown with partially purified preparations that betaine aldehyde dehydrogenase is quite specific, is DPN dependent, and requires —SH groups for maximal activity (619). Dinning *et al.* (620) studied the effect of diet on betaine transmethylase activity of rat livers and found that protein and amino acids had a marked effect on the activity of this enzyme system.

Studies with plant seedlings (568, 621) indicated the absence of a choline oxidase system, which would explain the known fact that betaine serves as a better methyl donor than choline in certain plants. More mature plants, however, apparently have a well developed choline oxidase system as evidenced by the formation of betaine from choline (568). In the tobacco plant the methyl group of choline is available for transmethylation, at least to nicotine (622). Unlike methionine, choline serves as a very poor methyl donor in yeast cells (623).

The preparation of highly purified choline acetylase was described (624), and the synthesis of acetylcholine in rats was reported to be decreased in deficiencies of thiamine and pantothenic acid (625). The subject of cholinesterases and anticholinesterases has been reviewed (626, 627).

Other metabolic studies.—Gradually a clearer picture of the methylation process is being developed, but it is far from complete. In an experiment in which rats were given a diet free of labile methyl groups but containing ample homocystine, B_{12}, and FA, du Vigneaud *et al.* (628) concluded that methyl group (and choline) synthesis occurred at a faster rate than when ample labile methyl groups were supplied in the diet. In studies on compounds serving as possible dietary sources of methyl groups in the rat, it was found that the methylsulfonium of methionine was well utilized and

that methionine sulfoxide was about 80 to 90 per cent as efficient as methionine (629); whereas, dicarnitine was inactive (630).

Hsu *et al.* (631) reported that in the chick the methyl groups of betaine were utilized more efficiently for tissue methionine formation than were choline methyl groups. In the mature fowl, choline can be used in the methylation of homocystine but not at a rate fast enough to meet the methionine requirement (632). Results with betaine were not given.

It is known that when a relatively large dose of choline is ingested by normal persons about 50 per cent is decomposed by intestinal bacteria to trimethylamine which is excreted in the urine. De La Huerga and co-workers (633, 634) showed that this decomposition was suppressed in man by oral administration of certain antibiotics or by feeding a lactobacillus culture. In dogs with damaged livers, as a result of phosphorus intoxication, large amounts of trimethylamine and low amounts of choline were excreted in the urine following doses of choline (635). In normal dogs ingested choline remained quite stable and was widely distributed in body tissues 4 hr. after feeding (636). Choline is absorbed from the intestine by way of the portal vein rather than the intestinal lymphatics as determined in studies with the dog (637). Artrom & Lofland (638) reported that rat liver homogenates have the ability to produce small amounts of trimethylamine from labeled choline. They found that trimethylamine formation was moderately depressed by fairly large amounts of choline or D-amphetamine but was increased by semicarbazide.

Rat studies.—Fischer & Garrity (639, 640) have studied the effect of a choline deficiency on serum composition. Using the sera of severely deficient animals, they found that the amount of albumin decreased, while phospholipide, α-globulin, and β-globulin increased. These changes appeared to be greater in the male rat than in the female and were influenced by age of the rat and stage of deficiency. The effects of starvation in the deficient rats on the blood pattern were not ruled out.

Other studies with the choline-deficient rat showed an increase in severity of deficiency signs by feeding L-cystine (641); the production of liver tumors in rats with combined deficiencies of choline, B_{12}, and FA (confirming earlier studies) (572); and changes in tissue concentration of Na, K, and Cl (642). Hall & Bieri (643) reported that 1 mg. of hydrocortisone injected daily prevented the typical renal lesions caused by choline deficiency, but control groups with pair-fed rats were not reported. Neither hydrocortisone nor growth hormone had any effect in preventing fatty livers. Wells (644) reported that feeding high levels of 2-amino-2-methylpropanol-1 and α,α-dimethyl-triethylcholine to rats produced more severe renal lesions than were produced by choline deficiency, but no effect on liver fat was noted. Choline administration counteracted the adverse effects.

Ward *et al.* (645) found that severe or prolonged choline deficiency in rats did not affect the degree of hydrolysis of fat in the intestine, the absorp-

tion of fat, or the level of serum lipides. No marked effects of x-irradiation on choline metabolism in rats were found in studies by Buchanan *et al.* (560). If large amounts of copper chloride are present in a diet, it may form two complex salts with choline which catalyze the oxidation of fat in the diet (646).

Mouse studies.—The feeding of 50 per cent of bentonite in a semisynthetic diet containing 6 per cent of yeast caused a retardation of growth, fatty livers, and liver tumors. The symptoms were prevented, in part, by dietary choline. Adsorption in the intestine of choline and probably other factors by the bentonite was suggested as the cause of the deficiency [Wilson (647)]. Mirone (648) studied the importance of choline in several strains of mice fed diets containing 23 per cent of casein and various levels of B_{12} and choline. With this high level of casein no effect of B_{12} or choline was found on growth up to 9 weeks. However, the importance of choline and B_{12} for normal reproduction and prevention of mortality of adult females and newborn mice was demonstrated. No effect of choline or B_{12} deficiency was found on the blood picture.

A depressing effect of cortisone on the reticuloendothelial system in mice was largely overcome by choline injections, but the diet used was not stated (649). In other studies mice fed a choline-deficient diet showed an increase in incidence of mammary tumors when treated with α-estradiol; but results with mice fed the same diet containing choline were not given (650).

Guinea pig studies.—A choline deficiency has been produced in the guinea pig for the first time by means of a semisynthetic 30 per cent casein diet [Reid (398, 651)]. Deficiency signs included poor growth, anemia, high mortality, pale and atrophic kidneys (not hemorrhagic), but no visible fatty infiltration of the liver. Approximately 0.12 to 0.15 per cent of choline was required in the diet for maximum growth. Neither betaine, homocystine, B_{12}, nor methionine substituted for choline. However, dimethylethanolamine is known to be equal to choline in its growth-promoting effect (652).

Additional studies with the guinea pig showed a partial protective action of choline against CCl_4 and ethionine toxicity (653, 654) and demonstrated that high levels of dimethylethanolamine, given by injection, were somewhat more toxic than equal levels of choline in both the rat and the guinea pig (655). Casselman & Williams (656) reported that a "centrilobular" type of fatty liver was produced in adult guinea pigs fed a semipurified diet, without added choline. The addition of choline to the diet prevented the deposition of fat. Since their diet appears to be low in methionine and since it contained certain ingredients usually considered as good sources of choline (1 per cent of liver powder, 7 per cent of "Cereograss," 12 per cent of extracted peanut meal), the experiment needs repeating with other types of diets before the specificity of choline can be proven.

Studies with fowls.—A detailed histological description of fatty livers of chronic choline-deficient chickens was given by Salmon & Copeland (572).

In some cases lesions considered to be hepatic malignancies were found, as in the rat. Also, increased amounts of hemosiderin (iron-containing pigment) in livers and spleens of deficient animals were seen. Histological deficiency signs in the cartilage of deficient chicks with "slipped tendon" were compared with those seen in manganese deficiency by Wolbach & Hegsted (657). Choline deficiency, with a resulting paralysis, was produced in the gosling for the first time by Briggs *et al.* (523). The deficiency signs appeared in only 3 to 5 days after the day-old goslings were placed on the diet.

Inexpensive concentrates of choline are now routinely added to many commercial poultry and swine rations by feed manufacturers. There is, however, only very limited evidence that commercial rations may be low in choline. The level of choline is apparently critical in rations for young turkeys (382), but it is very doubtful that commercial rations for growing chickens require additional amounts of this vitamin (658, 659). Although it is known that hens require a dietary source of choline (632), there is no evidence that added amounts of choline to normal laying or breeding rations are necessary for maximum results (343).

Other choline studies.—Hove *et al.* (660, 661) have described symptoms, for the first time, of a clear-cut choline deficiency in the rabbit. Deficiency signs (including poor growth, anemia, fatty and cirrhotic livers, necrosis of kidney tubules, creatinurea, progressive muscular dystrophy, and death) were prevented by about 0.13 per cent of dietary choline. Monomethylaminoethanol and methionine, when fed together, served as an effective substitute for choline, but there was no sparing action of B_{12} or FA.

Studies with choline in certain lower forms of life show that dietary choline is required by the parasitic dipteron *Pseudosarcophaga affinis* for larval growth and development (662); by larvae of *Tribolium confusum*, in quantities from 0.2 to 2.0 mg./gm. of diet (663); and by larvae of *Calliphora erythrocephala* (664). The tissues of the limpet, *Patella vulgata*, contained larger amounts of combined choline in summer than in winter (665); however, the significance of this is not known. Choline has been reported to stimulate the growth of the Reiter treponeme at relatively high levels (666) and to accelerate the regeneration of rhodopsin in liver-extirpated toads and frogs (667).

p-AMINOBENZOIC ACID (PABA)

Most of the studies made this past year with PABA have described its pharmacological properties and its resulting clinical uses, which are beyond the scope of this review. An excellent review on PABA appeared covering most phases of its metabolism and biochemistry [Harris *et al.* (667)].

Recent studies on this metabolite show that it is necessary for the growth of various strains of acetic acid bacteria (*Acetobacter suboxydans* and *Acetobacter melanogenum*) (668); for various lactic acid bacteria (in a few of which PABA is also inhibitory) (669); and for *Acetobacter xylinum* (670). PABA is equivalent to pantothenic acid in stimulating the growth of *Bacterium linens*

(671, 672), and it appears to be required for the conversion of α-ketoisovalerate to ketopantoate in this organism (673). Several studies have been made on the metabolism and growth-promoting action of many derivatives of PABA in bacteria (674, 675).

In spite of the known nutritional importance of PABA for various bacteria and certain other microorganisms, the reviewers suggest that this substance should no longer be regarded as a vitamin, but rather as a microbial nutrilite along with thioctic acid, various purines and pyrimidines, thiazole, adenylic acid, orotic acid, and many other compounds (676). In spite of earlier suggestions that PABA was a vitamin there is no evidence today that PABA is required for any higher animal, including man (667). No dietary deficiency of PABA has been reported in vertebrates even when fed the purest synthetic diets as long as ample FA is present. For instance, recent studies with the guinea pig show only a partial sparing action of PABA in the absence of PGA but no requirement in the presence of PGA (398, 521). Similar studies have been made with other animals (667).

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WATER-SOLUBLE VITAMINS, PART II[1,2]
(VITAMIN B_6, NIACIN, BIOTIN, ASCORBIC ACID)

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VITAMIN B_6

Vitamin B_6, as pyridoxal phosphate, is involved in a great variety of enzymatically catalyzed amino acid transformations: decarboxylation, transamination, racemization, dehydration, desulfhydration, cleavage and synthesis of tryptophan, cleavage of alliin, and cleavage of β-hydroxy-α-amino acids to glycine and aldehydes. The diversity of these transformations would at first glance seem to provide little basis for a common function for the coenzyme and would appear to place the catalytic burden on the protein. However, Snell and his co-workers, and others have succeeded in duplicating many of the above reactions by incubating the amino acid with pyridoxal, pyridoxamine, or a suitable analogue, and a metal salt at elevated temperature. Transamination (1, 2, 3) and racemization (4) of several amino acids, conversion of serine or cysteine to pyruvate (5, 6, 7), the reversible cleavage of threonine to glycine and acetaldehyde (7), the cleavage (8) and synthesis (9) of tryptophan, and the decarboxylation of histidine (10) have been accomplished in this manner. Braunshtein & Shemyakin (11) and Metzler, Ikawa & Snell (9) have proposed a general mechanism, involving a common intermediate, for the catalysis of these various reactions. The theories of the two groups are very similar. Braunshtein & Shemyakin (11) discussed the enzymatically catalyzed reactions and did not consider the role of metal ions. Metzler, Ikawa & Snell (9) based their discussion on the model studies which they have conducted and in which metal ions are known to play an important role. It is possible that the metal chelate forms are also the active ones in enzymatic catalysis, for kynureninase (12), cystathionase (13), and histidine decarboxylase (14) are stimulated by metal ions, and the inhibition of D-serine dehydrase by Cu^{++}, Co^{++}, or Zn^{++} is partially reversed by Mg^{++} (15). The structural requirements for vitamin B_6 activity are also in accord with the postulate that it functions as a metal chelate.

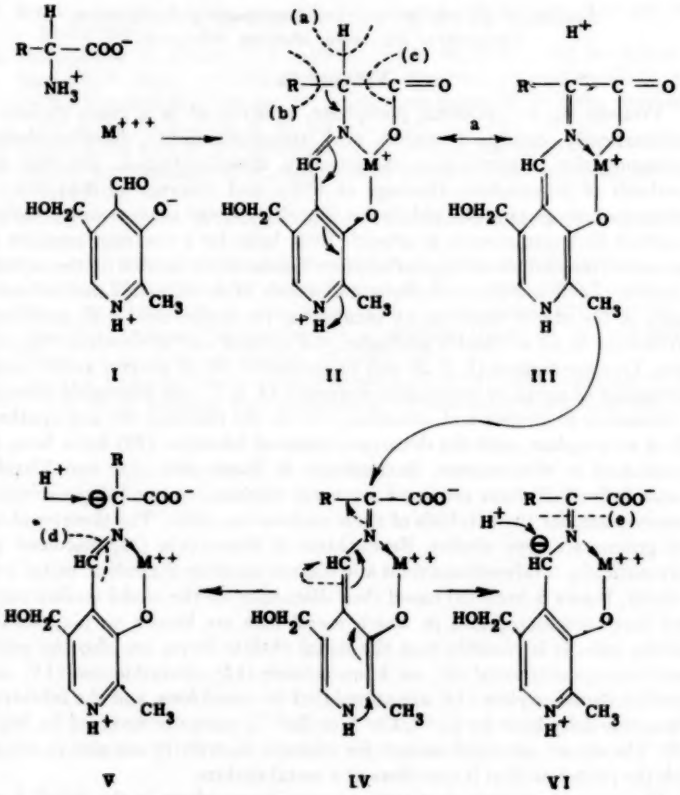
The theory proposes that the common intermediate is the Schiff base,

[1] The material in this review was selected from papers appearing before November 1, 1954.

[2] The following abbreviations are used: ADP for adenosinediphosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; INH for isonicotinic acid hydrazide; ITP for inosinetriphosphate; TPN for triphosphopyridine nucleotide.

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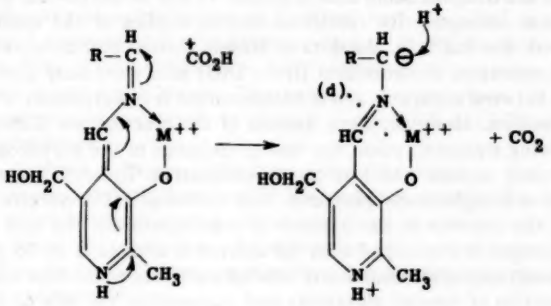
formed from the amino acid and pyridoxal, as its metal chelate, II. In this pyridinium structure, electrons are withdrawn from the α carbon atom of the amino acid skeleton. The chelated metal ion enhances this effect. Racemization could proceed by loss of a proton at (a) in II and its addition as in



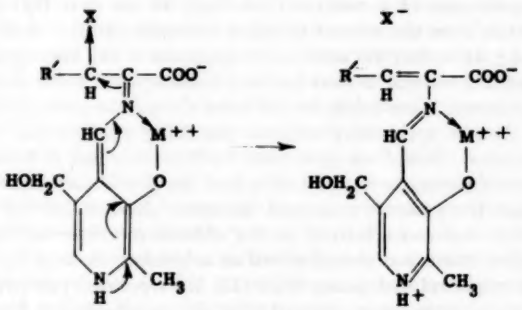
V followed by hydrolysis at (d). Transamination would result from hydrolysis at (e) in VI followed by reaction of the amine form of the coenzyme with an appropriate keto acid. The former reaction proceeds more rapidly at pH 10, the latter at pH 5.

Cleavage of II at (b) is exemplified in the enzymatic (16) and chemical (7) cleavage of hydroxyamino acids to glycine and aldehydes. Cleavage of II at (c) results in decarboxylation. Metzler *et al.* (9) depict this reaction as

proceeding through the sequence, II→VII⇄VIII where hydrolysis at (d) in VIII liberates the amine. In biological systems, without exception, the specific enzymatic decarboxylation of amino acids requires the presence of pyridoxal coenzyme (cf. 14).



VII VIII



IX X

If R in II contains an electron attracting group X on the β-carbon atom of the original amino acid as shown in IX, the sequence, II→IX→X, followed by hydrolysis of X results in liberation of ammonia, α-keto acid, and HX. Examples of biological reactions which parallel this are the dehydration of D-serine (15, 17) and L-threonine (18), the desulfhydration of cysteine, the well-known cleavage of cystathionine and related compounds, and the cleavage of tyrosine to phenol (19). Synthesis of tryptophan is postulated to

occur by reaction of serine with the coenzyme to stage VIII followed by addition of indole to the Schiff base of aminoacrylic acid.

The pathways of amino acid metabolism are therefore dependent on the nature of the R groups and most particularly on the specificity of the enzyme protein to which the complex Schiff base is bound. It will be worthwhile now to integrate these concepts with results of kinetic studies of the specific enzymes involved. For example, the data of Hilton, Barnes and co-workers (20) on the incorporation of deuterium (from D_2O) into glutamate during transamination between aspartate and α-ketoglutarate is in agreement with the above mechanism. However, some aspects of the reaction are difficult to interpret. During transamination, the rate of exchange of the α hydrogen on aspartate greatly exceeds the rate of transamination. In some manner the presence of α-ketoglutarate promotes this exchange. If aspartate is incubated with the enzyme in the absence of α-ketoglutarate the rate at which the α hydrogen is exchanged with the solvent is decreased by 95 per cent (20). Since only negligible amounts of labeled aspartate could have been produced by reaction of formed glutamate and oxalacetate, the role of the keto acid in the exchange reaction 'a' remains to be clarified.

The proposal of Metzler *et al.* (9) is in complete accord with data of Mandeles, Koppelman & Hanke (21) on the mechanism of amino acid decarboxylation. Bacterial enzymes which decarboxylate glutamic acid, lysine, or tyrosine do so with retention of the original α hydrogen atom. Experiments in a medium containing 99 per cent D_2O show that a single proton from the solvent is added stereospecifically, as in VIII. Cadaverine and γ-aminobutyric acid, in the presence of the appropriate decarboxylase, fixed D from the solvent but at a considerably slower rate than when these compounds were being formed from the parent amino acids.

A new pyridoxal-phosphate requiring enzyme has been found in extracts of *Clostridium sporogenes* by Wiesendanger & Nisman (22). The enzyme deaminates L-methionine and simultaneously cleaves the linkage between the γ-carbon atom and the sulfur atom to liberate methyl mercaptan which was characterized as the chloromercurimercaptide. The remaining carbon chain was characterized as α-ketobutyric acid by means of its 2,4-dinitrophenyl hydrazone. Wiss (23) has separated two pyridoxal phosphate-requiring enzymes concerned with the metabolism of kynurenine. Kynureninase occurs in the soluble fraction of liver proteins while a new enzyme, kynurenine transaminase, was found in liver mitochondria. The *o*-aminobenzoylpyruvic acid produced by the latter enzyme did not appear to be a precursor of anthranilic acid. Gooder & Happold (24) have discussed the role of pyridoxal phosphate in the tryptophanase reaction.

Pyridoxal, together with Cu, Co, Ni, or Fe salts, catalyzes the oxidative deamination (at 100°C.) of amino acids to yield the corresponding α-keto acids and ammonia (25). No biological significance was proposed for this finding. Riboflavin, which is known to function in the enzymatically catalyzed amino acid deaminations, was ineffective as a catalyst in the chemical

reaction, and sources of L-amino acid oxidase were found not to contain significant quantities of pyridoxal (25).

Detailed methods for the synthesis and isolation in crystalline form of the phosphoric esters of pyridoxamine, pyridoxal, pyridoxine, and deoxypyridoxine were reported by Peterson & Sober (26). Hurwitz (27) has studied the enzymatic phosphorylation of pyridoxal with a purified enzyme from yeast. In the presence of divalent cations, such as Mg^{++}, the enzyme catalyzes the reaction, ATP+Pyridoxal→ADP+Pyridoxal phosphate. It is therefore named pyridoxal kinase. The enzyme phosphorylates pyridoxamine and pyridoxine but is specific in accepting only ATP, not ITP or ADP[2] as phosphate donor.

Dietrich & Shapiro (28) have found that deprivation of dietary vitamin B_6 causes striking decreases in the cysteine desulfhydrase of mouse liver, considerable decrease in glutamic-aspartic transaminase, and slight decreases in dopa decarboxylase. Administering deoxypyridoxine caused similar decreases in the latter two enzymes but did not depress the cysteine desulfhydrase activity.

The extensive nutritional studies of vitamin B_6 by Beaton, McHenry and their co-workers have been summarized and integrated in a paper (29) which describes the time sequence of the metabolic alterations resulting from B_6 deficiency in the rat. One week after B_6 is removed from their otherwise complete ration (20 per cent casein, 20 per cent corn oil) rats have a significantly smaller amount of total body fat than pair-fed or *ad libitum*-fed rats on the complete ration. Significant differences in alanine-glutamic transaminase activity, and in urea formation by liver slices, were observed after three weeks of B_6 deprivation while differences in liver aspartic-glutamic transaminase activity were not significant until an additional three weeks had elapsed. Protein deposition was as good in the B_6-deficient animals as in the pair-fed controls. From these observations Beaton *et al.* drew the conclusion that B_6 deficiency affects, primarily, the rat's "energy production" thus depriving it of "surplus food for storage as fat" and assume that the effects of nitrogen metabolism are secondary. In view of the known function of B_6 in the metabolism of amino acids, particularly in the transamination (30) of amino acids not studied in this investigation, a more conservative interpretation should suffice. Since the ration contained an abundance of good quality protein there is no reason to expect a striking effect on protein deposition. However, a decreased ability to transaminate the great variety of amino acids would deprive the rat of a readily available reserve for fat and carbohydrate synthesis. To maintain its normal metabolism the B_6 deficient rat must call upon its fat reserves and hence these may be kept low (31). Tissue and hematological changes in B_6-deficient animals have been studied by a number of workers (32 to 37).

Vitamin B_6 deficiency in human subjects has been studied extensively during the past few years. Vilter *et al.* (38) induced the deficiency by feeding the antimetabolite deoxypyridoxine to 50 patients. Great variation was

noted between subjects in the time required for appearance of deficiency symptoms depending, in part, on the diet. Dermatitis, especially around the eyes and mouth, was the most frequently observed sign of deficiency. Glossitis and lesions of the buccal mucosa similar to that occurring in niacin deficiency occurred in several subjects. Disturbances of the nervous system were common, as would be expected from earlier work. These included nausea, lethargy, somnolence and confusion, and in a few patients, sensations of numbness in the hands and feet and impairment of vibration and position sense. Most of the patients receiving deoxypyridoxine excreted greater amounts of xanthurenic acid following the ingestion of 5 gm. of L-tryptophan than did control subjects. The patients responded rapidly to treatment with either pyridoxine, pyridoxal, or pyridoxamine in doses of 5 mg. or more daily. Skin lesions responded to local application of pyridoxine in an ointment. This is an exceptionally thorough study involving more clinical observations and laboratory tests than can be discussed here.

Deprivation of vitamin B_6 from the diet of two mentally defective children resulted in different deficiency symptoms (39). After four months, one developed a microcytic hypochromic anemia reminiscent of that first described by Fouts and Helmer in B_6-deficient dogs. The other infant experienced severe convulsions after having been on the deficient diet for 76 days. Convulsions, which subside upon administration of pyridoxine, have been reported by Malony & Parmelee (40) and Coursin (41) in large numbers of infants fed an artificial liquid milk formula without supplementation. It was found (41) that in the process of sterilization by autoclaving much of the pyridoxal and pyridoxamine in the formula had been destroyed. Heat treatment is detrimental to the B_6 content of solid foods as well, for Tappan, Elvehjem *et al.* (42, 43) find that both rats and monkeys require supplements of B_6 when fed a diet of natural foodstuffs which had been highly processed.

An unusual case of an infant requiring exceptionally large amounts of vitamin B_6 has been described by Hunt *et al.* (44). Convulsions began on the fifth day of life and disappeared during a period of general vitamin therapy. Repeated observations indicated that the seizures did not occur while vitamins were being administered daily but that on termination of treatment, convulsions would occur 48 to 56 hours following the last injection. The effective agent in the vitamin mixture was found to be pyridoxine. The infant showed no abnormality of xanthurenic acid excretion but, when observed at 23 months of age, its mental development and physical coordination were severely retarded. Although it is unlikely that there is a connection, it should be recorded that the mother had received injections of 50 mg. of pyridoxine three to four times a week from the second through the fifth months of pregnancy.

Sartori & Nico (45) reported infants to be resistant to the effects of deoxypyridoxine. Induction of a B_6 deficiency by feeding deoxypyridoxine seemed of little value in the control of acute lymphatic leukemia of adults, although one of four patients so treated appeared to have a brief remission of symptoms (46).

The question whether B_6 is of value in the treatment of nausea and vomiting of pregnancy has been disputed for a number of years. Pertinent to the discussion are the findings of Sprince *et al.* (47) and Wachstein and co-workers (48, 49) that pregnant women excrete higher than normal levels of xanthurenic acid, that test doses of tryptophan are not handled normally, and that administration of B_6 is helpful in restoring normal urine levels of tryptophan metabolites.

Isonicotinic acid hydrazide (isoniazid), which is used for the treatment of tuberculosis, gives rise occasionally to side effects which resemble in some respects the symptoms of B_6 deficiency induced by deoxypyridoxine. If the standard dose (2.5 to 3.0 mg./kg./day) is increased to 20 mg./kg. daily, 40 per cent of the patients develop peripheral neuritis after less than two months of therapy (50). Jones & Jones (51) found thiamine to be ineffective while niacin relieved the symptoms (dermatitis and mental changes) in one patient.

Biehl & Vilter (52) contend that the typical symptoms of isoniazid toxicity do not resemble those seen in pellagra, nor are they quite typical of those produced by deoxypyridoxine. They found that large doses (150 to 450 mg. daily) of pyridoxine would prevent the occurrence of neuritis in patients receiving the high dosage level of isoniazid. Growth retardation caused by feeding isoniazid to rats was prevented by feeding additional pyridoxine, but not by supplements of niacin (53). Pyridoxine supplements did not diminish the effectiveness of isoniazid in controlling tuberculosis in mice (53). Boone & Woodward (54) reported antagonism between isoniazid and vitamin B_6 in growing microorganisms, while Yoneda *et al.* (55) have found isoniazid to inhibit bacterial enzymes which require pyridoxal phosphate as coenzyme. Biehl & Vilter (52) found that patients receiving isoniazid excrete an excess of vitamin B_6 in their urine. They suggest that antagonism between these two substances in living organisms results when the hydrazide reacts with pyridoxal to form a hydrazone. The latter might be excreted and would be hydrolyzed in the assay procedure to liberate free pyridoxal.

Antagonism between vitamin B_6 and the pyrimidine moiety of thiamine, which produces convulsions and death in mice, was reported by Makino *et al.* (56). Kratzer *et al.* (57) have studied a heat-labile substance in linseed oil meal which inhibits growth of chicks. The growth inhibition can be counteracted by pyridoxine. Several analogues and homologues of vitamin B_6 have been synthesized (58) and used in biological and chemical studies. A strain of *Saccaromyces cerevisiae* which grows well on a medium of glucose, *p*-aminobenzoic acid, pantothenic acid, biotin, thiamine, and inositol was found by Moulder & Woods (59) to be inhibited by low concentrations of adenine or hypoxanthine. This inhibition of growth was augmented by niacin, niacinamide or, less effectively, by the pyridine nucleotide coenzymes. Pyridoxine at 10^{-9} to 10^{-10} *M* completely prevented the growth inhibition (50 per cent) produced by 10^{-6} *M* adenine and niacin.

In a series of papers Christensen, Riggs & Coyne (60, 61, 62) have re-

ported that pyridoxal (0.5 to 15 m*M*) enhances the ability of Ehrlich mouse ascites tumor cells, or of erythrocytes, to accumulate glycine and certain other amino acids. The higher concentrations (5 to 15 m*M*) of pyridoxal caused water and potassium losses from the cells. Deoxypyridoxine exerted similar effects on water and potassium shifts but inhibited glycine accumulation. A variety of acids (indolacetic, benzoic, phenylacetic, and kynurenic) produced effects similar to those evoked by pyridoxal. Tumor cells from B_6-deficient mice "had an inferior ability to concentrate glycine from 27 m*M* solutions and were stimulated by low levels of pyridoxal which were without effect on normal tumor cells."

Nicotinic Acid

Little progress has been made on the pathway of nicotinic acid formation from tryptophan in which the reaction sequence between 3-hydroxyanthranilic acid and nicotinic acid remains to be clarified.

In mutant strains of *Neurospora crassa*, which require either tryptophan or nicotinic acid, growth was stimulated by the known precursors of nicotinic acid, and in general, the growth response was poorer the further the compound was removed from nicotinic acid in the biosynthetic chain (63). Phenylalanine stimulated growth slightly, but its effect was greatly enhanced by the addition of *p*-aminobenzoic acid, which also potentiated anthranilic acid in some strains. *p*-Aminobenzoic acid is not a nicotinic acid precursor itself, but acts as a catalyst in an unspecified reaction. Surprisingly, its effect can be duplicated by low concentrations of sulfonamides. Newmeyer & Tatum (63) caution against drawing hasty conclusions about metabolic pathways from results obtained by blocked mutants.

Jakoby has studied the enzyme which hydrolyzes N′-formyl-L-kynurenine to liberate kynurenine in extracts of *N. crassa* (64). 5-Hydroxykynurenine has been synthesized by Butenandt *et al.* (65) and found to be inactive as precursor of the eye pigments of *Drosophila* or *Ephestia* as well as of nicotinic acid.

The pathways of kynurenine metabolism have been investigated further by Wiss (23), and two main mechanisms were found in rat liver: (*a*) removal of the side-chain and formation of alanine and anthranilic acid, and (*b*) transamination, leading to kynurenic acid formation. 3-Hydroxykynurenine is degraded by the same mechanisms. Kynureninase is present in the soluble liver proteins. Mitochondria contain the kynurenine-α-ketoglutarate transaminase but not kynureninase. Both enzymes require pyridoxal-5-phosphate as coenzymes.

The phosphoric ester of 3-hydroxyanthranilic acid has been postulated by Wiss & Hellmann (66) to be the immediate precursor of hydroxyanthranilic acid, and phosphorylation has also been suggested as the mechanism of introducing the phenolic group into kynurenine. However, the evidence for formation of hydroxyanthranilic acid phosphate is indirect, and experimental evidence will be required to establish the value of this interesting

hypothesis. Isotopically labeled 3-hydroxyanthranilic acid can serve as a precursor of N′-methyl nicotinamide in the rat (66a).

The nutritional requirements for nicotinic acid have been established for many species, and research is now directed towards clearing up the requirements of the remaining species and those prevailing under special conditions.

Briggs, Hill & Canfield (67) found that goslings require nicotinic acid when fed a purified ration, but they failed to establish the amount required for optimum growth or for prevention of hock deformities. Battig *et al.* (68) found that 85 per cent of the goslings reared on a practical chick starter diet developed perosis which could be prevented by raising the niacin content of the diet from 26 to 66 mg. per kg. Turkey poults on a variety of practical rations respond to supplements of nicotinic acid alone or in combination with other factors (69).

The breast milk of Bantu women on native, high-maize diets was found by Walker (69a) to contain only half as much niacin as that of similar women who consume a Europeanized diet. Differences in the tryptophan content of the diets are assumed to be the cause of the low concentration of niacin. The tryptophan content of the low-niacin milks was normal. Babies nursing mothers with the low-niacin milk grow as well as European breast-fed babies.

Fried & Carvalho da Silva (70) found that in rats depleted of protein reserves by feeding a nitrogen-free diet, niacinamide greatly increased the rate of weight recovery on an 8 per cent casein diet. Supplements of urea were also beneficial, and urea plus niacinamide gave somewhat better growth than either supplement alone. Combinations of nicotinamide and a nitrogen source such as urea or ammonium citrate raised the level of liver xanthine oxidase in young adult rats (70) and afforded protection against the toxic effect of sulfasuxidine in weanling rats (71). No protective action was observed in diets containing supplements of nicotinamide or nitrogen sources alone. These data may be interpreted as indicating a role for nicotinamide in utilization of simple nitrogen compounds for synthesis or sparing of protein (71, 72).

The effects of tryptophan deficiency were observed in adult rats kept on an acid-hydrolyzed casein diet containing nicotinic acid, and histological and chemical changes were recorded. Tryptophan deficiency affects all organs, especially the liver and endocrine glands; it causes a different pattern from that produced by pairfeeding or feeding protein-free diets to control animals (73) and can be clearly distinguished from the syndrome produced by nicotinic acid deficiency in the rat (74).

The site of electron addition to DPN[2] was established by Pullman, San Pietro & Colowick (75) by means of reduction of DPN with bisulfite in the presence of heavy water or enzymatically by alcohol dehydrogenase and deuterium-labeled ethanol. From the reduced DPN containing the isotope, the 2- and 6-pyridones were prepared. Deuterium was found in both the 2-pyridone and the 6-pyridone and hence cannot be attached in these two

positions. The authors conclude that the hydrogen atom attaches to the 4-position of the pyridine ring. (The stereospecificity of this hydrogen transfer is discussed in other chapters of this volume.) A quinoid structure is proposed for the reduced pyridine ring. Analogous results were found for methylnicotinamide, which is also reduced in the *p*-position (76).

Wang & Kaplan (77) purified a kinase from pigeon liver which forms TPN[2] from DPN. The enzyme phosphorylates the oxidized, but not the reduced, form of DPN. ATP is the specific phosphate donor, and the reaction is inhibited by ADP. The enzyme is different from that which forms CoA[2] by phosphorylation of its precursor. DPN phosphorylation is inhibited by reduced DPN and also by desamino-DPN at higher levels.

Hughes *et al.* (78) had found in 1952 that *Lactobacillus arabinosus* converts nicotinamide to nicotinic acid. The nicotinamidase presumed responsible for this conversion has now been obtained (79) in cell free extracts of several species of *Lactobacilli* and of a single strain of *Staphylococcus albus*. The enzyme could not be detected in several other types of bacteria. Highest activity was found in *L. arabinosus*. The enzyme is considered a constitutive, as opposed to induced, enzyme, for its activity does not depend on the composition of the medium and other external factors. No coenzyme could be found. Oka (80) has isolated a similar enzyme from yeast. Formation of nicotinamide from ammonium nicotinate has been observed in slices of rat liver and kidney by Quagliarello & Porcellati (81).

Methyl nicotinamide, which occurs in mammalian urine, is not found in the urine of insects (82). The level of nicotinic acid and its derivatives increases during the development of the silkworm pupa (83) and parallels oxidative activity. Rosenthal, Goldsmith & Sarett (84) measured the excretion of nicotinic acid metabolites in pellagra patients; methyl nicotinamide and methyl nicotinamide-6-pyridone were found to be lower than in normal humans. Methylation of nicotinamide is reduced in hyperthyroid rats. This may be related to the deficiency of vitamin B_{12} induced by hyperthyroidism, but no experiments were reported about the effect of vitamin B_{12} in this reaction (85). When nicotinic acid and glycine are incubated with rat kidney cortex mitochondria, nicotinuric acid is formed. This reaction requires an active oxidative phosphorylation (86).

Strength *et al.* (87) found that injection of aminopterin into rats lowers temporarily the level of DPN in their liver. The induced deficiency of folic acid containing cofactors possibly leads to reduced purine formation, and hence to lower levels of coenzymes.

Mizuno *et al.* (88) observed that the nicotinic acid antagonist, pyridine-3-sulfonic acid, can replace nicotinic acid for the growth of *Staphylococcus aureus* Terashima at concentrations of 10^{-4} to $10^{-2}M$ and enhances growth of *Shigella dysenteriae I* in the presence of nicotinic acid. This growth-stimulating action was attributed to the fact that pyridine-3-sulfonic acid inhibits diphosphopyridine nucleotidase; and this concept is corroborated by the fact that Neutral Red, another diphosphopyridine nucleotidase inhibitor,

likewise stimulates growth of bacteria. A similar mechanism was proposed for a series of nicotinamide derivates with alkyl or acyl substituents on the ring nitrogen atom. These compounds cannot replace DPN as coenzymes for dehydrogenases but can replace nicotinamide in some bacteria, although at only one-thousandth the activity of nicotinamide; however, it is claimed that contamination with nicotinamide can be ruled out (89).

Substituted nicotinamides were found by Hughes (90) to inhibit completely the growth of several bacteria. The most potent antagonist was 5-fluoronicotinic acid. The inhibition caused by fluoronicotinic acid can be reversed by nicotinic acid, nicotinamide, and pyridine nucleotides. The antagonist inhibits bacterial glycolysis, oxygen uptake, and cozymase synthesis but not cozymase uptake or function. It is probable that fluoronicotinic acid is taken up by the cells and metabolized in the same manner as nicotinic acid. Indeed, after incubation of cultures of *Streptococcus faecalis* with fluoronicotinic acid, a compound tentatively identified as fluoronicotinamide has been isolated. This is yet another example of incorporation of toxic compounds into coenzymes or other metabolites, a phenomenon which has been termed "lethal synthesis" by Peters (91). In *Lactobacillus arabinosus*, glycolysis is not inhibited by fluoronicotinic acid, but is stimulated by nicotinamide. This stimulation is blocked by fluoronicotinic acid. Nicotinic acid is more effective than nicotinamide or cozymase for reversing fluoronicotinic acid inhibition of growth and cozymase synthesis of *S. aureus* (90).

Two other nicotinic acid antagonists have been reported to be incorporated into higher metabolites by the same pathway as nicotinic acid. Zatman and co-workers (92) and Goldman (93) found that INH[2] is transformed by the organism into a nucleotide in which the nicotinamide moiety is replaced by isonicotinic acid hydrazide, thus forming an analogue of DPN. Another analogue of DPN, containing 3-acetylpyridine instead of nicotinamide, was isolated (94). The incorporation of acetylpyridine into this DPN-analogue was catalyzed by a diphosphopyridine nucleotidase from pig brain. This derivative of DPN is susceptible to reduction by alcohol dehydrogenase. The theory is advanced that bacteria are not inhibited by acetylpyridine, because they are devoid of this type of diphosphopyridine nucleotidase. Goldman (93) found that the INH-containing analogue was reduced neither by the usual chemical procedures, nor by DPN-linked dehydrogenases. The analogue does not interfere with the reduction of DPN or TPN by the respective reducing enzymes, nor with the oxidation of DPN-H by DPN-cytochrome-*c* reductase. Similar results were reported by Gutmann, Felton & Huennekens (95) who observed that INH and 1-isonicotinyl-2-isopropyl hydrazine inhibited only lactic dehydrogenase among a number of enzymes investigated.

A detailed procedure for the isolation of the INH-containing analogue of DPN and a description of its properties is given by Zatman *et al.* (96). The compound is formed by an exchange reaction analogous to that de-

scribed earlier for the reaction between DPN and nicotinamide (97), catalyzed by diphosphopyridine nucleotidase. INH and nicotinamide compete with each other for the enzyme complex. Incubation of the INH-nucleotide with nicotinamide leads to formation of DPN [cf. also (98)]. This exchange system is pictured in Figure 1.

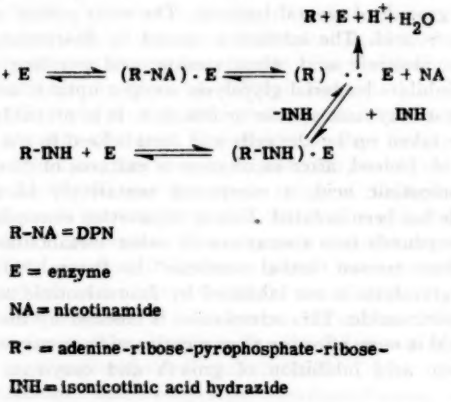


FIG. 1. Exchange system between diphosphopyridine nucleotide and its isonicotinic acid hydrazide analogue.

Kaplan *et al.* (99) suggest a mode of chemotherapy which is based on the exchange reaction between normal metabolites and suitable metabolic analogues. Such analogues would act by being integrated into compounds structurally similar to coenzymes or other metabolically important derivatives (cf. 91). The mode of action of 3-acetylpyridine was studied particularly; its toxicity derives from the fact that it is built into DPN-like compounds. In mice the toxicity of acetylpyridine is removed by administration of nicotinamide within the period of four hours before to two hours after acetylpyridine administration. DPN also protects against the drug, but nicotinic acid and tryptophan proved to be inactive; nicotinamide and DPN exert their protective action by blocking the formation of the acetylpyridine dinucleotide. After administration of acetylpyridine, the level of DPN in liver is increased, as is the level of nicotinamide excreted in urine. No acetylpyridine can be recovered from liver. It is concluded that the metabolic antagonist is metabolically transformed into the vitamin; this constitutes a novel mechanism of detoxication.

The acetylpyridine analogue of DPN can be isolated from several organs. It is present in highest concentration in neoplastic tissues, where the level of DPN is low. Kaplan *et al.* (99) suggest that incorporation of antagonists

may prove to be valuable not only for comprehension of exchange reactions, but also in chemotherapy.

Progress has been made in elucidating mechanisms of INH detoxication. In addition to the transformation into analogues of nicotinic acid-containing coenzymes, INH is hydrolyzed to isonicotinic acid; it is conjugated with glycine or acetic acid, forming 1-isonicotinyl-2-acetyl hydrazine and 1-isonicotininyl-2-glycyl hydrazine; and it also forms isonicotinic acid hydrazones of pyruvic acid and ketoglutaric acid. These compounds have been identified in the urine of rats and man (100 to 103). The acetyl derivative can be formed by incubating INH with cattle liver homogenate (100). Neuberg & Forrest (104) found that in the presence of INH, acetaldehyde accumulated in fermenting yeast as the isonicotinylhydrazone, while it was present only in traces in the absence of INH. The drug does not, however, interfere with carbon dioxide liberation or sugar consumption. INH forms chelates with a number of metal salts, and Albert (105) postulated that this may also contribute towards its mechanisms of action.

The determination of nicotinic acid by a modified König reaction, using sulfanilic acid for color development, has been proposed as the Official Method of the Association of Agricultural Chemists (106). Nicotinic acid and nicotinamide can be separated by means of an anion exchange column (Amberlite IRA-400) (107). Paper chromatographic procedures have been developed for the separation of a number of nicotinic acid derivatives (108) and isomers (108a) and for reduced and oxidized forms of pyridine nucleotides (109).

A method for the simultaneous determination of methylnicotinamide and pyridine nucleotides in blood has been developed (110) by combining removal of methylnicotinamide by cation exchange (111) with fluorimetric assay (112). The fluorescence of the condensation product of methylnicotinamide and acetone is considerably enhanced by additions of peroxide or metal salts, particularly those of cerium or iridium. The addition of these reagents may not precede condensation, else the fluorescence will be quenched (113). Oxidized and reduced DPN can be assayed simultaneously by a technique based on their different stability in acid and alkaline solution by means of the spectrophotometric method with alcohol dehydrogenase (114).

Plaut & Plaut (115) have prepared nicotinamide mononucleotide by an extension of Kornberg's (116) procedure. Following hydrolysis of DPN by pyrophosphatase the mononucleotide was purified by adsorption on charcoal, elution with amyl alcohol, passing through a Dowex-1 chloride column which retains adenylic acid and DPN, and finally by precipitation with acetone.

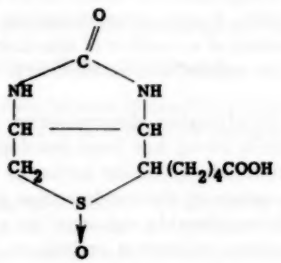
New derivatives of nicotinic acid have been synthesized for metabolism studies and as model compounds (117, 118). Four of a series of 41 pyrazine derivatives tested were found to inhibit growth of *Lactobacillus arabinosus* in competition with niacin. None was inhibitory, nor could they replace nicotinic acid, in rats and chicks (119).

The chemistry and biochemistry of pyridine nucleotide coenzymes have been reviewed (120).

Biotin

Melville (121) has prepared biotin sulfoxides from *d*-biotin by chemical oxidation and has resolved the mixture to obtain the two diasteriomers. Their biological activity varied greatly for different microorganisms, some of which transform the sulfoxide into biotin (122). The compounds did not stimulate growth in the biotin-deficient rat, but did prolong survival time. Like biotin, the sulfoxides can be bound by avidin. They did not shown anti-biotin activity. *d*-Biotin-*d*-sulfoxide has been isolated from milk as the methyl ester (121).

Wright & Cresson (123) have studied a new factor ("AN factor") which occurs in *Aspergillus niger* and which possesses biotin activity for *Neurospora crassa*. Wright, Folkers and their co-workers (124) have isolated the factor in crystalline form from *Aspergillus* cultures in which the yield of the factor was increased by adding pimelic acid. The compound was characterized (125) as *d*-biotin-*l*-sulfoxide, XI, by comparison with an authentic sample prepared by Melville (121).



XI. Biotin sulfoxide

When *A. niger* is incubated with desthiobiotin, biotin and biotin-*l*-sulfoxide are formed (126).

The behavior of several biotin derivatives in paper chromatography has been studied (127). When pure biotin is applied to the paper, two spots with biological activity for *Neurospora* can be isolated; these correspond in position to the two diasteriomeric forms of biotin sulfoxide. It is suggested that they arise from biotin as a result of oxidation by an unidentified component of the filter paper (Whatman No. 1). A discommodius usage of capital letter prefixes to designated rotations of compounds of unknown configuration occurs in the last two mentioned papers (126, 127).

Wright and co-workers (128) have found a relatively high biocytinase

activity in human blood. It could not be established whether this enzyme, which hydrolyzes biocytin to L-lysine and *d*-biotin, is identical with other hydrolases. Biotinidase, an enzyme which hydrolyzes bound biotin in peptic digests of liver, has been purified 20-fold from hog liver and separated from liver esterase by Thoma & Peterson (129). The enzyme hydrolyzes biocytin and *p*-(N-biotinylamino)-benzoic acid but not N-biotinyl-L-aspartic ethyl ester.

For the first time, biotin has been found useful in poultry rations under practical conditions. Robblee & Clandinin (130) found that turkey poults fed a starter ration composed of ground grains, alfalfa and cereal grass meals, meat scrap, soybean oil meal, herring meal, and mineral and vitamin supplements grew poorly, and a high percentage of the poults had diarrhea, perosis, dermatitis, and broken feathers. Adding 5 mg. pantothenic acid per 100 gm. of ration improved growth in early-hatched poults but did not alleviate the other symptoms. Biotin, at 50γ per 100 g. ration, prevented the deficiency symptoms and gave a large growth reponse in late-hatched poults when added to rations containing a supplement of pantothenic acid.

Several nutritional studies were concerned with the effects of sulfa drugs, antibiotics, and various vitamins on the intestinal synthesis of biotin (131, 132, 202). The study of biotin deficiency has been facilitated by a simple method of preparation of avidin (133).

Little has been added to the knowledge of the function of biotin. The known relationship between biotin, CO_2, and aspartic acid [see (134, 135)] has been extended (136 to 139). Thus, for *Lactobacillus arabinosus*, thymine can be replaced by aspartic acid in a synthetic medium; aspartic acid acts as a precursor of thymine. Aspartic acid can be replaced for this function by biotin and bicarbonate. Addition of biotin alone enhanced growth and partially spared aspartate, but bicarbonate alone was inactive. Threonine, lysine, and uracil, singly, or better combined, can also spare biotin. The inhibition of growth caused by cysteic acid can be counteracted by biotin or aspartic acid (138). Moat & Lichstein (139) have confirmed the finding of Winzler, Burk & du Vigneaud (140) that the fermentation and respiration of biotin-deficient yeast is greatly stimulated by biotin, particularly in the presence of a nitrogen source such as ammonia or aspartic acid. Moat & Lichstein (139) have shown that aspartic acid can replace biotin partially for this function. Since adaptation of biotin-deficient *S. cerevisiae* to sucrose fermentation was accelerated by biotin, the authors concluded that biotin may play a role in adaptive enzyme formation (139).

Chambers & Delwiche (137) have shown that extracts of biotin-deficient cells of *Propionibacterium pentosaceum* convert succinate to succinyl-coenzyme A as rapidly as extracts from cells grown on media containing biotin. Since the decarboxylation of succinate by deficient cells is greatly stimulated by added biotin, the vitamin is assumed to participate in the decarboxylation of succinyl-coenzyme A, either directly or by influencing the synthesis of the apoenzyme involved.

A role for biotin in nicotinic acid formation has been proposed by Sundaram, Tirunarayanan & Sarma (141). Biotin antagonists were found to block the biosynthesis of nicotinic acid in *Neurospora* and pea seeds, and this effect could be reversed by addition of biotin. In another study from the same laboratory (142), biotin was found to reverse the inhibition of *Neurospora* growth caused by the γ isomer of hexachlorocyclohexane, but only when a rather large amount of *myo*-inositol was present.

The growth of the biotinless mutant, *Escherichia coli* 58, in a medium containing aspartic acid to replace partially the biotin requirement, was inhibited by adenine and a variety of its naturally occurring derivatives and by other purines and pyrimidines (143). None of these compounds was inhibitory when biotin was added to the growth medium, nor were they inhibitory to the parent strain *E. coli* K12 which does not require biotin. When α-ketoglutarate, succinate, and other 4-carbon dicarboxylic acids replaced aspartate for growth of the mutant in the absence of biotin, adenine and its derivatives were less inhibitory, and the other purines and pyrimidines did not significantly affect growth.

Ascorbic Acid

To commemorate the two-hundredth anniversary of the publication of Lind's *Treatise of the Scurvy*, a symposium was held at the University of Edinburgh (144). The 19 papers read on this occasion dealt with the history and pathology of the disease and with various aspects of current research in this field. A careful investigation of the vitamin C requirements of human beings was summarized by Krebs (145). Less than 10 mg. of ascorbic acid per day was found sufficient to prevent or cure scurvy in young adult volunteers on an otherwise complete diet. The Medical Research Council's recommended intake of 30 mg. per day seems to provide an adequate margin of safety even though it is considerably less than the 75 mg. recommended by the U. S. National Research Council. However, in comparing these two figures, the different criteria employed by the two groups should be kept in mind (cf. 146).

The experimental study of ascorbic acid deficiency will be facilitated by the work of Woodruff *et al.* (147) and Reid & Briggs (148) who raised guinea pigs successfully on semisynthetic diets. Several years ago Briggs, Elvehjem *et al.* (149) found that ascorbic acid stimulated growth of chicks on purified diets and postulated that this effect was an indirect one, acting through the intestinal flora. March & Biely (150) have now observed similar effects when natural diets are used. The effect of added ascorbic acid depends on the nature of the basic diet, as well as the other supplements, such as folic acid, *p*-aminobenzoic acid, and antibiotics. Beneficial effects of ascorbic acid were observed especially in folic acid-deficient diets and were obtained even in the presence of antibiotics.

High amino acid excretion is found in scorbutic human adults and in babies, especially premature ones. Ascorbic acid administration causes a

greater fraction of excreted nitrogen to appear in the form of urea (151).

The two groups of investigators working at Columbia University and at Cambridge have continued to elucidate the main features of ascorbic acid biosynthesis in both plants and animals, The synthetic routes proposed by Isherwood, Chen & Mapson (152), and which were reviewed last year (153), have been investigated (154, 155) and from the evidence available two pathways were suggested:

(*a*) D-glucose → D-glucurono-γ-lactone → L-gulono-γ-lactone → L-ascorbic acid;
(*b*) D-galactose → D-galacturonic acid → L-galactono-γ-lactone → L-ascorbic acid.

Rats and cress seedlings can synthesize ascorbic acid from the lactone intermediate in both pathways. D-Mannono-γ-lactone is converted to D-araboascorbic acid (154). Pea and mung bean mitochondria oxidize L-galactonoγ-lactone to ascorbic acid. The open chain form of this acid was not converted to ascorbic acid, nor was L-gulono-γ-lactone (155, 156). Rat liver mitochondria, however, oxidize both the gulono and galactono lactones to ascorbic acid (157). These experiments suggest a transformation of hexose to ascorbic acid without change in the carbon skeleton of the molecule, and this is in agreement with the experiments of Horowitz & King (158, 159) with specifically labeled glucose. However, a pathway involving breakdown of the molecule and recombination cannot be ruled out entirely. L-Glyceraldehyde, but not the D isomer, inhibited ascorbic acid formation. This may indicate that glucose-6-phosphate, the formation of which by the hexokinase system is blocked by L-glyceraldehyde (160), is an intermediate in the biosynthesis of ascorbic acid.

Burns *et al.* (161) have determined the rate of ascorbic acid biosynthesis in normal and drug-treated rats by measuring the dilution of injected ascorbic acid-1-C^{14}. The body pool of ascorbic acid in normal rats is about 11 mg./100 gm. body weight; feeding chloretone or pentobarbital increases the pool to 19 mg. Normal rats were found to synthesize 2.6 mg. of ascorbic acid per 100 gm. of body weight while 12 and 22 mg. respectively were synthesized by pentobarbital and chloretone-treated animals. The increased excretion of ascorbic acid in rats treated with chloretone or pentobarbital (162) is therefore attributable to increased biosynthesis of ascorbic acid. The amount of ascorbic acid synthesized in the rat corresponds approximately to the amount necessary to produce tissue saturation in the guinea pig.

In the same study (161) it was found that only a part of the administered ascorbic acid was recovered as such from urine, ranging from 15 per cent in the normal to 40 per cent in the chloretone-treated rat. About one-third of the administered radioactive carbon was found in respiratory carbon dioxide. The remainder was excreted in the urine in the form of oxalate, dehydroascorbic acid, diketogulonic acid, and unidentified compounds. In man the amount of oxalate excreted in urine increased only after administration of more than four grams of ascorbic acid per day (163).

Biosynthesis of ascorbic acid in the rat is depressed by intake of low-protein diets. Under these conditions, chloretone stimulates biosynthesis and

brings ascorbic acid formation up to the level found in animals on high protein diets (164).

In necrotic rat liver the ascorbic acid content was reduced to zero, whereas the spleen of the same animals showed no change in ascorbic acid concentration (165). The addition of 1 or 2 per cent glucoascorbic acid to a Sherman-LaMer scorbutogenic diet did not produce visible deficiency symptoms in rats or mice, but after 20 days on the diet, the ascorbic acid level in plasma and several tissues dropped to zero (165). Feeding or injection of glucoascorbic had little influence on the growth of several tumors in mice and rats but retarded growth of liposarcoma in scorbutic guinea pigs (166). In mice glucoascorbic acid did not cure leukemia, but did prolong survival time (166).

Excess of dietary fluorine lowers the tissue ascorbic acid in rats and monkeys (167), a fact which was established for cattle in 1934 (168). Whole body x-ray irradiation lowers the urinary ascorbic acid, as well as the ascorbic acid levels of the organs, particularly the adrenals (169, 170). Two different strains of rats showed great quantitative variation in sensitivity to x-rays (170).

The gastric ascorbic acid in guinea pigs with gastritis was reduced as compared with the amount found in normal animals. It is suggested that the wound consumes locally more ascorbic acid than does normal tissue (171).

A new function for ascorbic acid has been reported by Udenfriend *et al.* (172, 173). By incubating aromatic compounds aerobically with ascorbic acid and ferrous or ferric salts at pH 6.7 and 37°C., phenolic groups are formed nonenzymatically. Adding a chelating agent enhances the rate of the reaction. The authors presume the reaction is brought about through the catalytic effect of an intermediary compound produced by the reaction of ascorbic acid with newly formed hydrogen peroxide. The reaction which occurs *in vitro* may well be simulated also *in vivo*, for the mild conditions under which it takes place are similar to those found in the organism. As a matter of fact, introduction of hydroxy groups into aromatic compounds foreign to the body is reduced in scorbutic guinea pigs and can be restored by administration of ascorbic acid (174). Keston & Carsiotis (175) successfully applied the technique of Udenfriend *et al.* to the hydroxylation of steroids, using three different compounds as substrate. The nature of the reaction product has not yet been established. Dalgleish (176) has recently extended this method of hydroxylation to other compounds including these of known physiological importance: tryptophan→5-(and 7)-hydroxytrpytophan; kynurenine→3-(and 5)-hydroxykynurenine; anthranilic acid→3-(and 5)-hydroxyanthranilic acid; phenylalanine→tyrosine, *o*-tyrosine, and several dihydroxyphenylalanine isomers.

According to Uchida *et al.* (177) the first step in the oxidation of *p*-hydroxyphenylpyruvic acid, a metabolite of tyrosine, to homogentisic acid, is the formation of 2,5-dihydroxyphenylpyruvic acid. The enzyme that catalyzes this reaction was purified from rabbit liver. When dialyzed, the enzyme

becomes inactive but can be reactivated by the addition of ascorbic acid and vitamin B_{12}. The authors imply that vitamin B_{12} acts as a coenzyme. However, the quantity of B_{12} required for maximum activity (10 γ in a few ml.), and the fact that ferrous ion in combination with ascorbic acid was also active, indicate that vitamin B_{12} may have served only as a nonspecific source of a metal. The requirements for this reaction, which involves both hydroxylation and migration of the side chain, resemble those described in the preceding paragraph. The oxidative decarboxylation of 2,5-dihydroxyphenylpyruvic acid to homogentisic acid requires another enzyme and supplements of DPN and cocarboxylase (177).

The role of ascorbic acid in the transformation of folic acid to citrovorum factor has been studied in chick liver homogenate (178) and in extracts of bacteria (179). Both formate and ascorbic acid are required for maximum conversion by *Streptococcus faecalis* cells under anaerobic conditions (179).

The addition of 5 or 10 per cent of tyrosine to the diet of chicks resulted in poor growth, abnormal feathering, and high mortality. This toxicity could be partially removed by supplementing the diet with ascorbic acid, but not with folic acid (180).

Scurvy is known to alter collagen formation and metabolism, e.g., the collagen of scorbutic animals has been reported to contain much less chondroitin sulfate than does normal collagen (181). Reddi & Nörstrom (182) found that in scorbutic guinea pigs the rate of incorporation of labeled sulfate into chondroitin sulfate was one-third that found in normal animals. They suggested that the rate of sulfate incorporation could be used as an index of ascorbic acid nutritional status.

According to Becker *et al.* (183), the rate of cholesterol synthesis is increased in scorbutic guinea pigs. Scurvy is accompanied by a decreased cholesterol content in the adrenals, spleen, and lungs, an increase in testes and small intestine, and no change in liver and kidney (184).

Exposing rats to cold (−6°C.) reduces the liver glycogen level to a minimum after 24 hr. Administration of 150 mg. ascorbic acid daily hastened the rate of recovery slightly (185).

The interrelation of ascorbic acid and various hormones, particulary those of the adrenal cortex, continues to be studied intensively. The level of 17-ketosteroids in the blood (186) and urine (187) of scorbutic guinea pigs is much greater than normal. The high rate of ketosteroid excretion can be enhanced by administration of ACTH (187). ACTH does not reduce the level of ascorbic acid in the liver nor does it hasten the onset of scurvy (188). Cortisone likewise is without effect on the symptoms of scurvy in the guinea pig (189). These results are in agreement with studies summarized by Kark (190) in which it was found that the stress of arctic cold did not significantly increase the ascorbic acid requirement of men. Likewise, there was no evidence that ascorbic acid deficiency curtailed adrenal function.

The present status of the relationship between ascorbic acid and hormones has been reviewed by Lloyd & Sinclair (191).

Vitamins in General

Nurmikko (192) has studied the relation between growth factors by growing two different bacteria symbiotically on a medium which is deficient for either organism alone. A similar, but less extensive experiment has been reported by Doctor & Couch (193).

For nutrition experiments it is of interest that vitamins were found in beet sugar and maize dextrose (194).

The feeding of vitamins, beef extract, or liver mitochondria had no effect on the survival time of eviscerated rats (195).

The incidence of spontaneous tumors in *Drosophila* is increased by 50 to 100 per cent by increasing the amount of vitamins added to the growth medium (196).

The following subjects have been reviewed: bacterial growth factors by Woods (197), vitamins in human nutrition by Wright (198), the function of vitamins and their place in therapy of mental disorders by Peterman & Goodhart (199), the symptoms of nutritional deficiency in man by Thomson & Duncan (200), the relationship between vitamins and antibody formation by Axelrod & Pruzansky (201), absorption of vitamins from the gut by Chow *et al.* (202). A new book on the biochemistry and physiology of nutrition, edited by Bourne & Kidder (191), contains several chapters on vitamins and coenzymes. Sebrell & Harris (203) have edited a monograph, in three volumes, on all the vitamins. One noteworthy feature is that the editors of this book do not make a division between water- and fat-soluble vitamins on the grounds that no such distinction is physiologically justified. On the other hand, Kodicek (204) stresses the importance of the division of vitamins into these two main divisions and proposes the name "prosthetin" for the water-soluble vitamins, to denote their function as coenzymes. It seems unlikely that an additional name for the water-soluble vitamins will prove useful.

The Food and Nutrition Board of the National Research Council has published a revision of *Recommended Dietary Allowances* which includes recommended intakes for several of the vitamins (205).

The Vitamin Society of Japan is publishing a new quarterly, *The Journal of Vitaminology*.

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WATER-SOLUBLE VITAMINS, PART III[1]
(THIAMINE, LIPOIC ACID, RIBOFLAVIN, PANTOTHENIC ACID, INOSITOL, AND MISCELLANEOUS FACTORS)

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In 1954, as in past years, the interest of biochemists in the vitamins has been primarily centered on function. It is becoming increasingly possible to interpret life processes in terms of molecular reactions, and it is at the level of molecules reacting with molecules that the mode of action of the B vitamins is being sought. Yet when one surveys the literature published in 1954, it is evident that the majority of papers are concerned with practical problems.

In this review the available literature has been covered from the last review (1) through October, 1954. Although the emphasis will be on the function of the vitamins at the molecular level, an attempt will be made also to cover the nutritional and clinical literature.

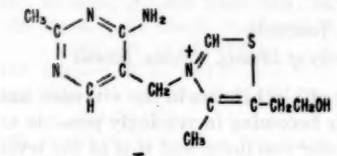
THIAMINE

Chemistry.—The disulfide form, III, of thiamine, I, was first prepared by Zima & Williams (2) and found to be equal to thiamine in biological activity by Zima, Ritsert & Moll (3). The discovery of allithiamine, V, by Fujiwara *et al.* (4) has renewed interest in this subject. Allithiamine and its propyl homologue, VI, were both more active than thiamine for the growth of rats on a B_1-free diet and were curative when given to B_1-deficient animals. They were absorbed more readily than thiamine from the intestine and caused a marked elevation in thiamine blood levels in both animals and humans. A greater urinary B_1 excretion following oral allithiamine than after an equivalent amount of thiamine, indicated better absorption of the disulfide forms (5, 6).

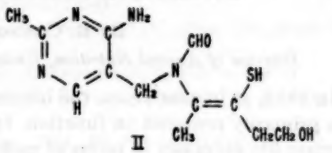
The group at Takeda Pharmaceutical Industries, Osaka, under Taizo Matsukawa (7 to 24) have continued their long series of investigations on thiamine derivatives. They have found (23) that allithiamine is formed by the reaction of thiamine and alicin, the latter arising from the enzymic de-

[1] The following abbreviations are used: ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); E for enzyme; FAD for flavin-adenine-dinucleotide; fl for riboflavin; FMN for flavin mononucleotide; GSH for glutathione; MPT for monophosphothiamine; NA for nicotinic acid; PA for pantothenic acid; PABA for p-aminobenzoic acid; PCMB for *p*-chloromercuribenzoate; PGA for folic acid; PP*i* for inorganic pyrophosphate; T for thiamine; TPT for triphosphothiamine; THFA for tetrahydrofolic acid.

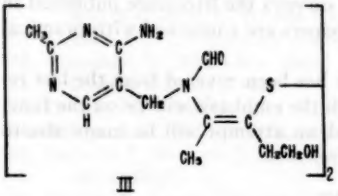
composition of alliin by allinase as shown in Figure 1. This type of reaction was also confirmed for the methyl and propyl thiamine disulfides, VII and VI, by using synthetic S-methyl and S-propyl-L-cysteine sulfoxides and allinase isolated from the plant. Thus they confirmed the presence in the plant of alliin and the S-methyl and S-propyl-L-cysteine sulfoxides. Marked



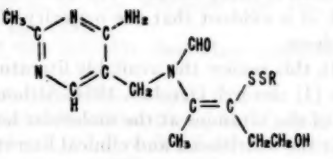
I



II



III

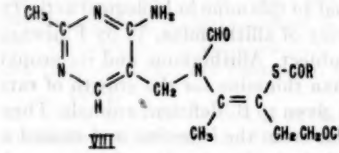


IV

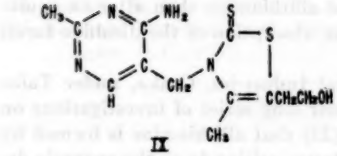
V - R=-$CH_2CH=CH_2$

VI - R=-$CH_2CH_2CH_3$

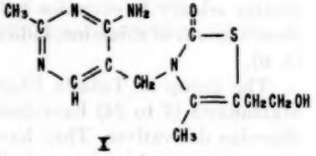
VII - R=-CH_3



VIII



IX



X

differences were found between extracts of different plant species (19, 20), varying from no alkyl disulfide, to 1, 2 or even 3 homologues. L-Cysteine readily reduces thiamine disulfide, III, or the mixed disulfides, V, VI and VII, to form thiamine (thiol type, II, and cystine or mixed cystine disulfides (S-alkyl mercapto-L-cysteine) (21, 22). Heating the disulfide forms in ethylene glycol, or similar high boiling solvents, converts them to thiochrome (7, 11).

For the synthesis of alkyl disulfides of thiamine various dialkyl disulfides were oxidized with H_2O_2 in glacial acetic, and the alkyl alkylthiosulfinates formed were reacted with thiamine at pH 8. Eleven disulfides, from methyl

to benzyl, were prepared (8, 10) and 24 O-acyl alkyl disulfides (14) (acyl esters of the $-CH_2CH_2OH$). All were found equal to thiamine in biological activity. Similar alkyl disulfides were also prepared from homothiamine (ethyl instead of methyl at the 2 position on the pyrimidine ring) (10). Thiamine aryl disulfides have been prepared by application of arylsulfenyl chloride etc. to the thiol type of vitamin B_1 (15, 25).

By application of acylating agents (e.g., benzoyl chloride, acetic anhydride, etc.) to thiol-type thiamine, II, a number of O, S-diacyl compounds,

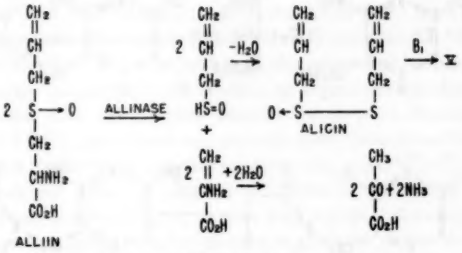


FIG. 1. Allithiamine biosynthesis.

VIII have been prepared (12, 13, 24). Again all were found approximately equal to thiamine in biological value; however, when thioethers were prepared (18) none showed any appreciable biological activity. Apparently either a free or readily formed $-SH$ (as from the disulfides) is required for biological activity. This gives some support to a role of this SH group in the biological function of thiamine (14) which will be discussed later.

B_1 synthesis.—Since the formation of thiamine from its thiothiazole (SB_1), IX, is almost quantitative (26) and the synthesis (27) of the SB_1 is simple, this is claimed to be one of the best procedures for thiamine synthesis (26, 28, 29). SB_1 is as active biologically as is thiamine (27), and it is readily converted to thiamine disulfide (9) and to the thiazolone (X) (17). A new derivative ($-$tetrahydrofurothiazole-2-thione), XI, an intermediate in certain SB syntheses (Figure 2), has been synthesized (16, 30); its=S is replaced by=O on reaction with H_2O_2 in acid or alkali while a thiochrome derivative is formed by H_2O_2 in neutral solution (31).

Nesbitt & Sykes (32) have developed a good method for the preparation of N-methylthiamine (the 4-methylamino-pyrimidyl derivative) for analogue studies. This compound had been shown to possess B_1 activity by Schultz (33). The biologically inactive compounds, chlorothiamine and bromothiamine, have been reported in samples of commercial synthetic thiamine (34). Roux & Couzinie (35) have used hydrated phosphorus oxychloride for the synthesis of polyphosphoric esters of thiamine, polyphosphate residues being attached to both the amino and the hydroxyl groups.

Assay.—A number of reports have appeared during the year on new B_1 assay methods (36 to 40), as well as many modifications of existing procedures

(41, 42, 43). New Methods have included (*a*) Biological—the use of the rice-meal beetle (*Tribolium confusum*) (36) not only for thiamine, but also for riboflavin, pantothenic acid, etc.; (*b*) Microbial—the use of *Escherichia coli* K_{12} mutant on a simple medium and sensitive to 10 μμg. by the cup-plate method with a useable assay range of 0.1 to 1000 mμg./ml. (37); and (*c*) Chemical—for large amounts as in pharmaceuticals, Yoshimura (38) has split the thiazole ring in H_2SO_4 by liquid Zn amalgam under CO_2, and the

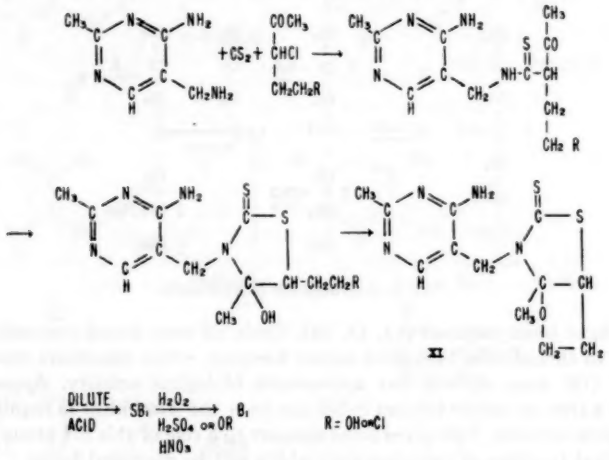


FIG. 2. Thiamine-thiazolone synthesis.

H_2S, which is quantitatively evolved, is absorbed in $CdSO_4$ solution and titrated with $Na_2S_2O_3$ after adding excess KI + I. The method is for milligram amounts of B_1. Another approach is that of Bandelin & Tuschhoff (39) who precipitated the thiamine as the reineckate and read the red color of the acetone solution. Also for milligram amounts polarographic methods can be used (40, 41). The thiochrome procedure, however, is still most generally popular, and elimination of extraneous fluorescence by the use of a special photometer results in an assay range of 0.0025 to 1 μg./ml. (42). Interference is also reduced if the oxidation is carried out at pH 10 in a boiling water bath for 5 min. (43). For higher levels of thiamine as in pharmaceuticals, optical density measurements of the thiochrome at 370 mμ, rather than reading the fluorescence, improves the accuracy although decreasing the sensitivity (10 to 200 μg.) (44).

Chromatography and electrophoresis.—A number of reports have been devoted to the separation of thiamine derivatives by chromatographic methods (45 to 58). In addition, these technics have been used as tools in much of

the work on thiamine, as for example, that of the Japanese groups previously cited. Thiamine is readily separated from the other B vitamins by paper chromatography (45, 46, 47) and can be quantitatively determined by spot length (48). The separation of the various phosphorylated derivatives of thiamine has been carried out with a number of different solvent systems (49, 50, 51). In the work of Siliprandi & Siliprandi (51) thiamine was readily separated from its phosphoric esters by chromatography on filter paper in 10 to 100 μg. amounts and on ion exchange (Amberlite 1RC-50) columns in milligram amounts. Starch columns were only useful for the separation of T and MPT from DPT and TPT[1] (i.e., the enzyme active from the enzyme inactive forms). By paper electrophoresis (ionophoresis) (see also 52, 53, 54) they were able to separate up to 2 mg. amounts of thiamine and its phosphoric esters, while even larger amounts could readily be separated by electrophoresis on cellulose powder columns. It was possible to prepare chromatographically pure DPT and TPT, and these compounds were then found to be enzymatically equal on a molar basis to yeast cocarboxylase (cf. 55). The technics used in this work should prove invaluable to future investigators. In view of the physiological importance of pyruvate in vitamin B_1 deficiency, the quantitative separation of a large number of α-keto acids by paper electrophoresis of their dinitrophenylhydrazones should also prove useful (56, 57, 58).

Thiaminase.—Thiaminase, the enzyme from fish, fern, etc. which splits thiamine, has been recently reviewed by Fujita (59).

Metabolism of thiamine.—In work on the over-all metabolism of S^{35}-thiamine (60) and 2-C^{14} thiazole-labeled thiamine (61, 62, 63), it was found that the urine is the main excretory pathway. Only small amounts of expired $C^{14}O_2$ were found and only small amounts of radioactivity appear in the feces. By paper chromatography of the urine of rats receiving C^{14}-labeled thiamine, 16 different compounds were separated. In addition to traces of phosphorylated forms of thiamine and larger amounts of thiazole derivatives, there were a number (8 total) which gave a positive thiamine response with *Lactobacillus fermenti*. In view of the work already mentioned on the disulfide forms of thiamine, it would appear quite possible that these compounds are mixed disulfides of thiamine being excreted by the body. Sahashi *et al.* (64) have reported the interaction of thiamine disulfide and pantetheine to produce B_1—S-S-pantetheine and similar reactions with glutathione and other SH compounds.

DPT is dephosphorylated in the brain by a specific enzyme, thiamine dephosphorylase, which has been localized histologically in the nerve cell bodies (65).

Studying the phosphorylation of thiamine, Bartley (66) found that in respiring kidney particles P^{32}-labeled orthophosphate was slowly incorporated into a cocarboxylase substrate, but that the tissue suspensions were unable to synthesize DPT by orthophosphate phosphorylation of MPT or T or to synthesize MPT from T. He speculated that "lipothiamide" (67) is the

reactive form of thiamine and that phosphorylation occurs on the lipothiamide, which then breaks down to DPT or MPT. However, Rossi Fanelli *et al.* (68), studying thiamine phosphorylation in the intact rat, found that ATP^1 labeled with P^{32} was a precursor of DPT and TPT, but not of MPT, and Cerecedo *et al.* (69, 70), using a purified enzyme preparation from rat intestinal mucosa and from rat liver for DPT synthesis, found ATP and Mg were required. Neopyrithiamine inhibited this synthesis of DPT, while oxythiamine had no effect, yet *in vivo* feeding of oxythiamine to pigeons resulted in decreases in tissue DPT (71). In an interesting nutritional experiment Orabona (72) was able to cure vitamin B_1 deficiency in pigeons with thiamine unless the birds had been on diets containing excessive amounts of folic acid (10 mg./day) or nicotinic acid (15 mg./day), in which case thiamine was inactive in curing B_1 deficiency, but DPT was curative, indicating that the excess folic and nicotinic interfered with the *in vivo* phosphorylation of thiamine. Magyar, applying his previous work on riboflavin (73) to the other B vitamins, including thiamine, states that phosphorylation is essential for absorption (74). However, the body is able to phosphorylate thiamine or MPT to cocarboxylase when T is injected directly into the blood stream, although DPT and TPT did give a more immediate, sharp increase in blood cocarboxylase activity than did T or MPT (75). TPT is not split at once to MPT and DPT in the body as it is *in vitro*.

With increasing intakes of thiamine, increasing amounts are found in the liver up to an intake of 2 mg./rat/day, at which level the liver contains 11 to 16 μg./gm. wet weight, with manganese apparently increasing liver B_1 (76). On reversing this and depleting rats of thiamine, the lowest levels of thiamine are reached first in the muscles, while normal concentrations are maintained in the brain for a longer time (77, 78). It is possible, however, to reduce the thiamine content of the brain, particularly by the use of the antagonist neopyrithiamine (79). In addition to the usually considered pathways of excretion, thiamine has also been found to be excreted in sweat in small amounts (about 1 μg./ml.) (80). Surprisingly homogenates of the kidney or liver of rabbits appear able to synthesize thiamine *in vitro* from 4-methyl-5-β-hydroxyethyl thiazole and 2-methyl-4-amino-5-aminomethyl (or chloromethyl) pyrimidine (81). Under the same conditions, rat or pigeon, liver or kidney homogenates (82) had no such synthetic activity.

Functions of thiamine.—Wright & Scott (83) have found that in B_1-deficient tissues of rats, pyruvate oxidation is depressed and can be regenerated by the addition of DPT, while in the same tissues α-ketoglutarate oxidation is not greatly lowered and DPT was not stimulatory. While the mechanism is not clear, they suggest that DPT is more tightly bound by α-ketoglutarate dehydrogenase than by the pyruvate decomposing enzyme and that high fat diets alleviate B_1-deficiency symptoms because of oxidation of fat bypasses pyruvate oxidation. The finding of methyl glyoxal in the urine of vitamin B_1-deficient rats and its complete absence in the normal rat's urine (84) is difficult, at present, to explain in terms of the known function of

thiamine in pyruvate metabolism. The apparent reason for the build-up of methyl glyoxal in B_1 deficiency is the marked reduction in liver glyoxalase,

$$\left(CH_3CHOHCO_2H—H_2O \xrightarrow[GSH]{\text{glyoxalase}} CH_3COCHO \right)$$

in the deficient animal even though GSH[1] and not DPT is the coenzyme for this enzyme (85). Salem (84) restates the suggestion of Vogt-Møller (86) that the symptoms of thiamine deficiency may be those of methyl glyoxal intoxication. This has also been said of pyruvic acid (87).

The mechanism of action of DPT (as discussed below) in all cases involves the formation of an aldehyde-enzyme complex or "active-aldehyde" which will undergo various reactions including transfer and also various condensations. For example, in pig heart and pigeon breast muscle DPT catalyses the formation of acetoin (acetyl methyl carbinol) from pyruvate (88, 89) while a bacterial system will carry out several DPT dependent condensations using various substrates and forming, in addition to acetoin and acetic acid, also diacetyl methyl carbinol (from diacetyl) and α-acetolactate (from pyruvate) (90, 91).

The role of vitamin B_1 in the metabolism of alcohol has been of interest to nutritionists for years. Work by Hulpien *et al.* (92) indicates that vitamin B_1 is involved in alcohol metabolism at the stage of the utilization of acetaldehyde ("active aldehyde" formation). They observed that EtOH at dosages which produced only mild intoxication in normal dogs, produced severe illness or death in dogs pretreated with the B_1 antagonist, oxythiamine. The increased toxicity was accompanied by a marked fall in blood glucose. The same results were obtained when acetaldehyde was given by slow intravenous infusion to oxythiamine-treated dogs, but again, as with EtOH, it produced no effects on normal dogs, indicating that the role of B_1 in EtOH metabolism is at the acetaldehyde utilization stage and not in the primary oxidation of EtOH.

However, Vitale *et al.* (93) found no difference between the thiamine-deficient and the thiamine-normal rat in the metabolism of ethanol-1-C^{14} to $C^{14}O_2$. In both cases about 80 per cent of the alcohol was metabolized to CO_2 in 7 hr.

Mechanism of thiamine catalyzed reactions.—Matsukawa *et al.* (94) have shown that O, S-diacetyl thiol thiamine readily transfers the acetyl attached to the S to aniline or glutathione as shown in Figure 3. Matsukawa & Kawasaki (12) have postulated that in the body S-acetyl thiamine pyrophosphate undergoes transacetylation acetylating CoA to form acetyl CoA. While this mechanism could be useful in explaining the clastic reaction in which acetate and formate are produced from pyruvate via acetyl DPT (95), it does not account for the role of DPT as "aldehyde carrier" and the separation of decarboxylation from dehydrogenation (96) in the oxidative decarboxylation of pyruvate. On the other hand, their suggestion and that of Karrer and

Viscontini (97) that the thiol form may be the "active form" of DPT is given support by the high biological activity of the thiol and disulfide forms of thiamine and the high coenzyme activity of Thiol-DPT (98). Oh this basis a mechanism in which the S of the thiol form of DPT, XII, is the "carrier group" has been combined with the reaction sequence proposed by Gunsalus (99) to give the formulation shown in Figure 4. An —SH dependent thiamine coenzyme such as this should be inhibited by *p*-chloromercuribenzoate. This was found to be true (100), but it was concluded from the relief of the inhibition by monothiols, but not by T or DPT, that a site other than thiamine—possibly lipoic acid also required by this animal pyruvic dehydrogenase—was the site of PCMB[1] inhibition. The recent observation that

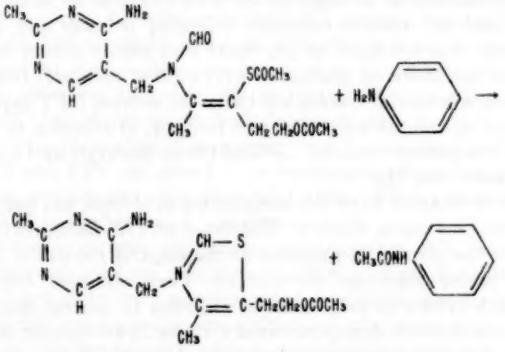


FIG. 3. Transacetylation from O,S-diacetyl thiamine.

PCMB at $10^{-5}M$ inhibits the pyruvic carboxylase (yeast type) and acetoin forming activities of two bacterial systems, *Pseudomonas lindneri* and *Acetobacter suboxydans*, which are DPT but not lipoic acid mediated (101) strongly indicates that the PCMB acts at the thiamine stage. The latter does not differentiate between a reaction with DPT-SH and enzyme (protein) SH.

Mizuhara & Handler (88), studying the formation of "active acetaldehyde" (90) in the diacetylmutase and carboxylase systems, have proposed essentially the same formulation except that in their case the tertiary nitrogen of DPT-pseudo base, XIII (tautomeric with DPT-thiol), rather than the S of the thiol form, coordinates with pyruvate (or diacetyl) to form an intermediate which decarboxylates (or loses a ketone-like group) to leave an "active acetaldehyde" carrying molecule. This scheme is shown in Figure 5.

The only difference in the two systems (Figures 4 and 5) is whether the N or the S carries the aldehyde group. In either case the reaction can be visualized as a decarboxylation to yield an "active aldehyde" which may then react in various ways, depending on the organism or enzyme system, to

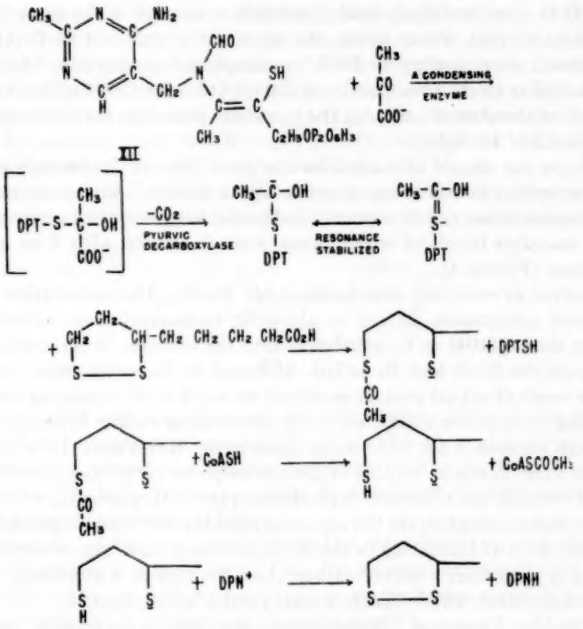


FIG. 4. Mechanism of thiamine action.

yield acyl lipoate as in the pyruvate system of *E. coli* (96), to yield acetaldehyde in the *A. suboxydans* system where the decarboxylation reaction precedes the oxidation (102), to yield acetoin by condensation, to yield by oxidation acetyl DPT or acetate or in the usual mammalian system (as given in

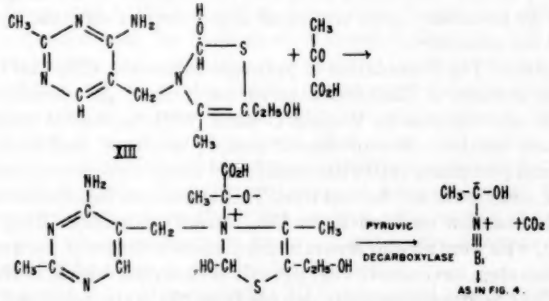


FIG. 5. Mechanism of thiamine action.

Figure 4) to acetylate lipoic acid either free or as part of the same molecule (lipothiamide) (67). From lipoic, the acetyl is transferred to CoASH and the electrons are picked up by DPN^1 to complete the oxidation. This scheme appears similar to the formulation of Calvin (103) for CO_2 fixation in photosynthesis at the step of carrying the pyruvate over into the citric acid cycle (a step blocked by light).

Perhaps one should also consider the possibility of the tertiary nitrogen of the pyrimidyl amino group functioning as aldehyde activator, since this would require more nearly neutral conditions; however, the energy required for the transfers involved would seem more favorable with S as acceptor throughout (Figure 4).

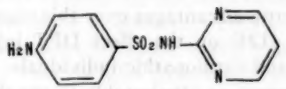
Thiamine in microbial metabolism.—(*a*) Yeasts: The stimulation by thiamine and ammonium sulfate or alcoholic fermentation of baker's yeast has been shown (104) to be attributable to the increase in carboxylase synthesis from the N, S, and B_1 added. Although in the early stages this may not be a result of actual protein synthesis as much as of supplying coenzyme to existing apoenzyme (105), soon the ammonium sulfate is supplying the precursors necessary for continued apoenzyme formation. In a study of thiamine-dependence in strains of *Saccharomycetes cerevisiae*, cocarboxylase was destroyed in the thiamine-dependent strain during growth, while in the thiamine-independent strain the cocarboxylase level remained constant. This was interpreted as indicating in the B_1 dependent strain, the occurrence of a very active degratory enzyme rather than the loss of a synthetic enzyme (106), a suggestion which clearly awaits further investigation.

(*b*) Molds: *Phycomyces blakesleeanus* synthesizes β-carotene, and this synthesis is inhibited in medium containing less than 0.2 μg. thiamine/100 ml. (107). On the other hand, growth inhibition begins at B_1 levels 10 times as high at 2 μg./100 ml. The effect of B_1 may be a reflection of its role in "active aldehyde" condensation reactions and may indicate the mechanism for polyene synthesis in the plant. Surprisingly, Schopfer *et al.* (108) found that small amounts of neopyrithiamine (1 to 10 mμg./25 ml.) increased carotene synthesis by *Mucor hiemalis*. Perhaps in this organism the antagonist can serve as a B_1 precursor (some strains of *Mucor* require only the pyrimidine portion of the molecule).

(*c*) Bacteria: The degradation of pyruvate to acetate, CO_2, and hydrogen by cell-free extracts of *Clostridium butylicum* [termed the phosphoroclastic reaction for this organism by Wolfe & O'Kane (109), because of its similarity to the *E. coli* reaction which produces "acetyl phosphate" and formate from pyruvate and phosphate (110)] has been found (109) to require cocarboxylase as well as coenzyme A and ferrous iron. This appears to be another modification of the reaction mechanism for DPT already discussed. King & Cheldelin (102), who were able to separate the decarboxylation of pyruvate from the oxidative step, have shown that pyruvic carboxylase which, in the case of *A. suboxydans* produces free acetaldehyde from pyruvate, requires DPT and Mg^{++}.

(*d*) Protozoa: Sulfadiazine (2-sulfanilylamino-pyrimidine), XIV, at 5 to 12 mg. per cent of the medium can replace thiamine in supporting at least partial (½ normal) growth for the thiamine-requiring organism *Tetrahymena geleii* (111) while other sulfonamides cannot. Thiamine activity of sulfadiazine had previously been reported for the rat (112). No hypothesis is offered to account for such activity, but it might possibly mean that the tertiary 2-pyrimidyl N acts as an acetyl acceptor-activator.

Thiamine in animal nutrition.—Of the large number of papers reviewed in this field only a few can be mentioned. The injection of neopyrithiamine and



XIV

oxythiamine into the egg increased mortality, and chicks which hatched showed typical neural symptoms (113). Rats also showed disturbances in embryonic development in thiamine deficiency with many fetuses stillborn or resorbed (114, 115). With two weeks B_1 deficiency prior to breeding, 90 per cent of the rats resorbed (114).

Chickens fed a diet completely devoid of thiamine developed abnormal electrocardiograms in 7 to 10 days (116), the abnormalities including bradycardia, depression of S-T segment, sinus arrhythmia, sinus arrest, and auriculo-ventricular dissociation with nodal rhythm.

Because of the role of thiamine and pantothenic acid in acetyl transfer, Bose *et al.* (117) studied the effect of these two separate nutritional deficiencies on the synthesis of acetylcholine in the rat. While either deficiency produced an appreciable decrease in the acetylcholine content of the heart, brain, and particularly liver, the thiamine deficiency produced the greatest effect. Casella & De Carlo (118) have reported that thiamine deficiency in the rat reduces the rate of conduction of the action potential by nerve, although Berry *et al.* (119) found that nerves from B_1-deficient cats conduct impluses normally, and the findings of Mannell & Rossiter (120) indicate no effect of thiamine deficiency (acute or chronic) in the rat on nerve myelin sheath condition, nor on myelin lipides, nor on nerve pentose nucleic acids.

In continuation of their work on the instability of thiamine in "synthetic" rations, Waibel *et al.* (121) have found that salts, particularly K_2HPO_4, but also $CaCO_3$ and $MnSO_4$ are the ingredients most responsible for the rapid destruction of thiamine. One per cent glycerol markedly reduced the disappearance of thiamine from the ration under a wide variety of conditions. However, where it is necessary to know accurately the thiamine intake, it should probably be given in solution by dropper.

Working with pigs, Moustgaard (122) has reported that no rise in blood pyruvate occurs if the animals have been trained to lie still for 40 to 60

min. before taking the blood sample. If excited, normal animals showed elevated blood pyruvate and lactate. The elevated blood pyruvates found at times in thiamine-deficient animals are possibly attributable to their greater excitability.

In human nutrition.—Perhaps of greatest interest has been the studies on the toxicity of thiamine. Thiamine shock has been described in laboratories in various parts of the world, and the mechanism of the toxicity which has been generally attributed to anaphylaxis, following large intravenous doses, has more recently been attributed to ganglionic blockage (123).

A number of clinical reports have indicated the feeling that DPT may, at times, have therapeutic advantages over thiamine. One such example is that of Barbieri *et al.* (124) on the effect DPT injection on the electrocardiogram in normal and cardiopathic individuals. They report beneficial results which they interpret as attributable to the direct function of DPT in muscle metabolism.

A strain of *Staphylococcus aureus* which was isolated from the duodenum of an individual with idiopathic steatorrhoea and polyneuritis may be related to the etiology of beriberi. Washed cell suspensions of this organism in glucose and thiamine were able to remove 4 μg. of thiamine per ml. from the solution within 1.5 hr. (125). The B_1 was not destroyed, but was stored in the cells, a situation reminiscent of the thiamine-depleting effects of yeast (126).

Caster *et al.* (127) have refined their method of "pyramin" determination and re-emphasize the usefulness of this pyrimidine excretion as an index of thiamine status in the individual and as an improved tool for nutrition surveys. From surveys it is evident (128) that beriberi is still a disease of worldwide importance; however, there is hope that proposed rice-enrichment programs will one day wipe out the disease.

Thiamine interrelationships.—Various dietary ingredients "spare" thiamine in that animals receiving them grow and survive on low-thiamine diets which would otherwise prove fatal. Among these agents are the antibiotics. Penicillin, for example, promotes the well-being of rats and chicks on a low-thiamine ration. On the basis of bacteriological studies on the coliform organisms of the feces and cecal contents of rats in penicillin low-B_1 experiments Balakrishnan *et al.* (129) arrived at the conclusion that the role of the antibiotic is in the elimination of thiamine-utilizing bacteria from the tract, thereby making available to the host more of the thiamine of the diet. *In vitro* a penicillin sensitive strain of *Sarcina lutea* has been found to require thiamine (130). Such a hypothesis would lead one to the conclusion that the actual nutritional requirement in terms of body needs of B_1 is considerably below the dietary requirement. On the other hand, Schendel & Johnson (131, 132) found that in the rat dietary penicillin markedly increased the growth of rats on suboptimal levels of thiamine whether the vitamin was given orally or by injection [in contrast to the chick (133)], although the antibiotic gave no growth stimulation when adequate thiamine

was administered (orally or by injection). This finding would favor the hypothesis of increased intestinal synthesis of the vitamin [in the case of pantothenic acid versus chlortetracycline (aureomycin), exactly the same findings were obtained]. Certainly all workers agree that the animal on the low B vitamin plus antibiotic diet grows better because he is getting more of the vitamin. More is found in the feces, in the urine, and in the carcass. Evidence from the increased dilution of fed radiothiamine would also tend to support the increased synthesis theory (134). Very similar results are obtained by feeding high levels of ascorbic acid to rats on low B_1 diets (135). In an inverse relationship vitamin B_{12} has been found to greatly accelerate the appearance of polyneuritis in older rats (120 gm.) fed a B_1-low ration (136). Evidence has also been obtained that cortisone is antagonistic to thiamine and DPT and that its administration increases the requirements of the animal for B_1 (137, 138).

Lipoic Acid (thiotic Acid)

Lipoic acid has been covered in a series of excellent reviews in a symposium on the metabolic role of lipoic acid (139). Consequently this subject will not be reviewed except for the paper of Franken & Stapert (140). These workers found that the oxidation of pyruvate by pigeon breast or pigeon heart muscle homogenates, which is impaired in B_1 deficiency, responded to *in vitro* DPT addition if K_3FeCN_6 was provided as the hydrogen acceptor, but not in the case of oxygen. In the latter case it was necessary to add a supernatant prepared by centrifugation of normal muscle homogenate in addition to DPT. This is interepreted as indicating loss of lipoic acid along with thiamine in the deficient animal, which could be construed as indicating that lipoic acid normally occurs in the tissues as lipothiamide pyrophosphate.

Riboflavin

Microbial synthesis.—The work on the pathway of riboflavin synthesis initiated by MacLaren (141) and others has been continued during the present year. Using the organism *Ashbya gossypii*, Plaut in a continuation of his earlier work has shown that formate carbon is a precursor of carbon 4 and that glycine is the precursor of the fused ring carbons 4a and 9a. He has pointed out that this pattern is analogous to the established pattern of precursors for the purines as shown in Figure 6 (142). The purine pathway originally postulated by MacLaren (141) is further expanded by the work of Goodwin & Pendlington (143) using *Eremothecium ashbyii* (144). They have proposed the synthesis scheme given in Figure 7 on the basis that factors added to the medium which stimulate markedly riboflavin production are probably building blocks for riboflavin synthesis. Threonine or serine or both were markedly riboflavogenic as were the purines, xanthine adenine, and adenosine. The effect of stimulatory amino acids was not additive, while that of an amino acid plus a purine was. On the other hand, pathways similar

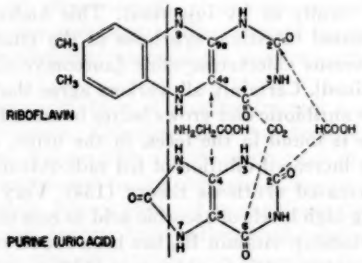


FIG. 6. C^{14} incorporation into riboflavin and purine.

to those used by the organic chemist for riboflavin synthesis or by *E. coli* for cobalamin synthesis were apparently not related to riboflavogenesis in *E. ashbyii* and probably other organisms, since neither 5,6-dimethylbenzimidazole, nor 1,2-dimethyl-4-amino-5(D-1′-ribitylamino) benzene stimulate riboflavin snythesis. Interestingly the purine nucleosides and nucleotides are not, in general, as effective as are the free purines in increasing the yield of riboflavin (145), indicating that they are not directly involved in the metabolism of *E. ashbyii* as immediate precursors of riboflavin. In support of the scheme given in Figure 7 the radiocarbon is principally "lost" in the

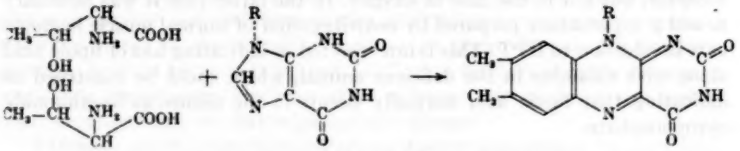
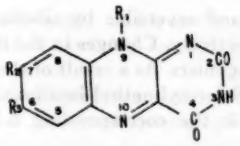


FIG. 7. Route of riboflavin biosynthesis.

process of adenine 8-C^{14} going to riboflavin as compared to uniformly labeled adenine to riboflavin. The pathway in *S. cerevisiae* may be somewhat different, in that while the purines stimulated riboflavin synthesis, so did many pyrimidines (146); however, this may merely indicate an ability to make from them a ribityl diamino pyrimidine such as might also be derived from adenine (145). In contrast, also, this organism was inhibited, both as to growth and in riboflavin production, by serine (as well as tryptophan and phenylalanine) which was a stimulant in the work of Goodwin *et al.* (143).

Plaut (147) has also investigated possible precursors of the aromatic and ribityl portions of riboflavin. This work has shown that acetate (carbons 1 and 2) (also 148) and glucose (carbons 1 and 6) will both serve as riboflavin precursors, the carboxyl of acetate labeling carbons 6, 7, 8a, and 10a, XV, while glucose 1 or 6 and acetate-methyl label principally carbons 5, 8, and the methyls (Figure 8). The possibility is suggested that 2-carbon frag-



	R_1	R_2	R_3
XV —	$CH_2(CHOH)_3CH_2OH$	CH_3	CH_3
XIX —	$CH_2(CHOH)_3CH_2OH$	H	C_2H_5
XX —	$CH_2(CHOH)_3CH_2OH$	CH_3	Cl
XXI —	$CH_2(CHOH)_3CH_2OH$	Cl	CH_3
XXII —	CH_3CHO	CH_3	CH_3
XXIII—	CH_3CH_2OH	CH_3	CH_3

ments may be the basic units involved in the biosynthesis of ring A of riboflavin (149).

Chemical synthesis.—A chemical synthesis of FAD[1] has been reported (150). By the condensation of salts of the cyclic ester of riboflavin-5′-phosphate, XVI, with 2′,3′-isopropylidene-adenosine-5′-benzyl-phosphoryl-chloride, XVII, in warm phenol, followed by removal of the isopropylidene residue, P′-adenosine-5′-P²-riboflavin-5′-pyroposphate, XVIII, identical with the natural coenzyme, flavine-adenine-dinucleotide (FAD) was obtained (Figure 9).

Antivitamins and structural analogues.—Lambooy and co-workers in a continuation of their studies on riboflavin analogues have prepared 6-ethyl-

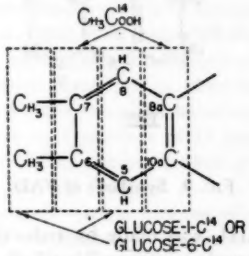


FIG. 8. C^{14} incorporation into ring A of riboflavin.

9-(D-1′-ribityl)-isoalloxazine, XIX, and found it to possess 3 per cent of the activity of riboflavin for *Lactobacillus casei* (151). Apparently while methyl groups at 6 and 7 are not essential for activity, both positions filled give greater activity since the diethyl and 6-ethyl 7-methyl derivatives are essentially equal to riboflavin for *L. casei*.

They have also prepared 6-chloro-7-methyl-9-(D-1′-ribityl) isoalloxazine, XX, and the 6-methyl-7-chloro compound (XXI, and found them both to be

antagonists for *L. casei* and reversible by riboflavin (152) with inhibition indices of 85 and 22 respectively. Changes in the ribose part of the molecule have also resulted in antagonists. As a result of the degradation of the ribose side chain, 6,7-dimethyl-9-formylmethyl isoalloxazine, XXII, was obtained in high yields and from it the corresponding 6,7-dimethyl-9-(2′-hydroxy-

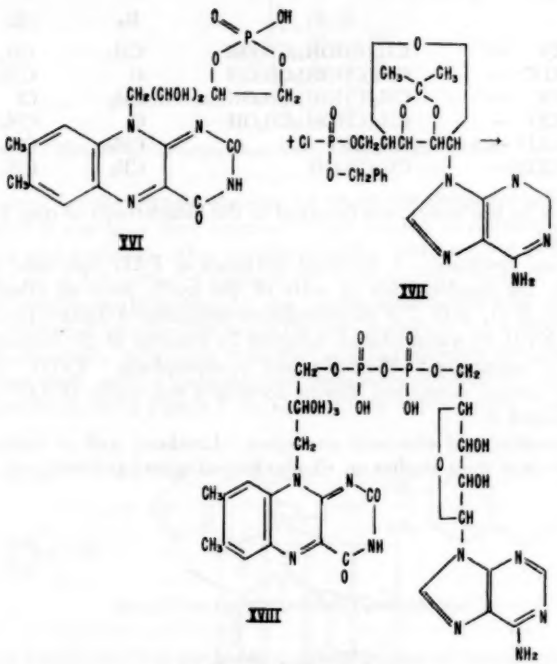


FIG. 9. Synthesis of FAD.

ethyl) isoalloxazine, XXIII, was made by reduction with sodium borohydride (153). These compounds are reversible riboflavin antagonists, not only against *L. casei*, but also for rats.

Occurrence, forms of riboflavin.—Povolotskaya (154) has reported the existance of a "new" protein-bound form of riboflavin which is released by trypsin. It is not determined by the usual chemical or microbial riboflavin assay methods, although reported to be widely distributed and present in amounts equal to the sum of FMN, FAD, and riboflavin. The existence of such a compound would necessitate revision of assay procedures and existing assay data. A new derivative has also been reported in liver extracts heated above 90°. This compound moves faster than FMN[1] and FAD, but slower

than riboflavin on butanol-acetic acid-water paper chromatograms (155). This compound, in addition to being formed by heating FAD, was also formed by some bacteria.

The association of the protein-coenzyme in the "old yellow enzyme" has been studied using a photomultiplier-amplifier-recorder system (156) sensitive to 10^{-9} mole free FMN. It is stated that dissociation may be initiated by splitting between the amino group of FMN and protein, whereas combination is initiated at the FMN phosphate to protein bond.

A derivative of riboflavin, riboflavinyl glucoside, is produced by a transglucosidase of rat liver according to the reaction: riboflavin + maltose → riboflavinyl-α-D-glucoside + glucose. The same enzyme will synthesize the glucosides of several isoalloxazine derivatives, including many which are antivitamins (157). The reaction is competitively inhibited by α-D-glucose-1-phosphate. The riboflavinyl glucoside is much more water-soluble than is riboflavin and could conceivably be of importance in riboflavin transport in the tissues; however, the compound has not yet been shown to occur naturally.

Assay methods.—Antibiotics which might well be present (because of their widespread use in animal feeding) in samples to be assayed for riboflavin have been shown not to interfere with the *L. casei* assay for riboflavin. Apparently the autoclaving eliminates any inhibition (158). By increasing the glucose (to 3 per cent) and the buffer capacity of various media for the *L. casei* assay of riboflavin, Clarke (159) has markedly increased the range of the assay and has obtained linear log-dose response curves which improve the statistical interpretation of the assay.

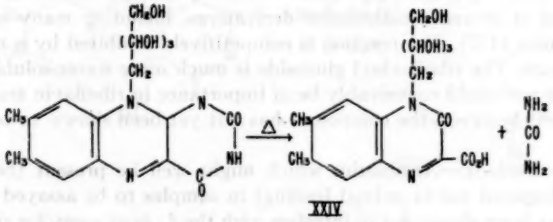
Separation methods, chromatography, and electrophoresis.—By paper electrophoresis Siliprandi *et al.* (160) have separated riboflavin, FAD, and FMN. The electrophoresis was carried out in acetate buffer pH 5.1 for 6 to 7 hr. and the spots located under ultraviolet by their bright yellow fluorescence. This system also permitted the complete separation of the flavin, B_6, and NA[1] coenzyme forms in a total mixture of 11 compounds. However, riboflavinyl glucoside has a mobility similar to that of riboflavin and cannot be separated easily by this method (161).

By paper chromatography with a number of different solvent systems (160), FMN and Fad were separated from one another and from the other phosphorus-containing compounds, ATP, ADP, AMP,[1] and DPN, and it was shown that in the phosphorylation of ADP to form ATP,[1] FAD is not a carrier of phosphate for the oxidative phosphorylation (162, 163), a situation which had been confused previously by incomplete separation of other phosphates from FAD. Using chromatography on a powdered cellulose column, Whitby (164) has developed a relatively simple procedure for the preparation of FAD. The yields obtained were excellent (3.3 mg./kg. bakers yeast), and the purity quite good (60 to 80 per cent).

Chemical properties, stability studies.—Riboflavin is unstable to light, to alkali, and even to heat. Considering illumination, it has been found that the

decomposition was greater with light of wavelengths within the range 365 to 590 mμ and that there was no decomposition above 610 mμ (165). An intermediate between riboflavin and lumichrome on photolysis in acid and one between riboflavin and lumiflavin on photolysis in alkali have been demonstrated by paper chromatographic means and detection under ultraviolet, or by the red spots obtained with ammoniacal $AgNO_3$ (166). β-Mercapto propionaldehyde (methional) has been found to be a product of an illumination-catalyzed reaction between methionine and riboflavin (167).

Considering alkali and heat destruction, Farrer & MacEwan (168) have shown first that riboflavin is stable for 1 hr. at 80°C. for pH 1.3 to 6.5; 1 hr.



XXIV

FIG. 10. Thermal destruction of riboflavin.

at 100°C. for pH 1.7 to 5.5; or 1 hr. at 120°C. for pH 2.0 to 5.0; and second, that the products of thermal hydrolysis are ammonia, urea, 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid, XXIV (Figure 10), and an unidentified compound. Thermal destruction thus gives products similar to those obtained by alkaline breakdown (169) and quite different from those obtained by photolysis. At very acid pH values thermal destruction was merely a reversible reduction, fluorescence being regenerated by aeration (168).

Metabolism of the vitamin.—Again, as in the case of thiamine (78) brain flavin coenzyme content was found to be essentially independent of dietary riboflavin intake (170). However, in other tissues coenzyme concentration varied with dietary vitamin intake. The intake necessary to maintain coenzyme levels at optimum values varied with the tissue and the coenzyme, but in general, 30 μg. of riboflavin/day/rat was sufficient for maximum FAD and FMN. However, for optimum D-amino acid oxidase activity an intake of over 60 μg./day/rat was required. For optimum growth 43 μg/rat /day of riboflavin was required but, in general, tissue coenzyme levels reached maximum at lower levels of intake than were required for optimum growth rate.

Functions of riboflavin.—Riboflavin functions as an electron carrier in many oxidation enzymes. These may function directly or indirectly as diagrammed in Figure 11 (171). Work has been carried out on a number of these enzymes this year, including studies on xanthine oxidase, cytochrome-*c*

reductase, aldehyde oxidase, succinic dehydrogenase, nitrate reductase, hydrogenase, choline oxidase, and menadione reductase.

Xanthine oxidase, an FAD-containing enzyme, contains molybdenum as an obligate part of the enzyme (172, 173). It appears that the Mo or Mo plus FAD is present in a particular, probably chelate form, in that while addition of supernatant (which contains 90 per cent of the Mo plus FAD originally present) following dialyses reactivates the apoenzyme, addition of sodium molybdate gives partial reactivation only at extremely high levels of Mo, and FAD addition has no further effect (174). Because of the rapid response of xanthine oxidase (liver or intestinal) to dietary changes in

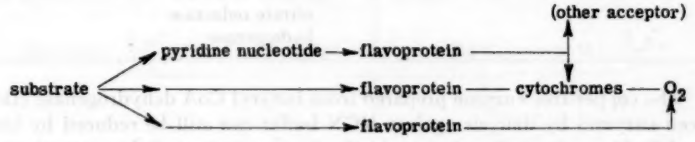


FIG. 11. Role of flavoproteins in electron transport.

protein [level or quality (175)], there has been considerable interest in assay methods for xanthine oxidase. A rapid colorimetric method for xanthine oxidase activity based on the use of 2,6-dichlorophenolindophenol as hydrogen acceptor and its subsequent photometric absorption is only useful for purified enzyme preparations (176). A colorimetric method (177) which can be applied directly to tissues and which is sensitive enough that tissues of very low xanthine oxidase activity may be as easily analyzed as tissues of high enzymatic activity is based on the conversion of xanthine to uric acid and the conversion of the uric acid to allantoin by the tissue uricase. The sum of the uric acid and the allantoin formed, determined colorimetrically, is the index of xanthine oxidase activity; the amount of allantoin can be used at the same time as an index of uricase activity. In liver only allantoin need be determined because of the abundance of uricase in this tissue. A new manometric method (178) has also been proposed which does away with the previously obtained high endogenous oxygen consumption values by inhibition with pyrophosphate with no effect on the xanthine oxidase system. This method is sensitive and relatively quick and gives results in good agreement with values obtained by xanthine disappearance.

One of the components of choline oxidase (choline→betaine) has been found to be FAD (179). A new enzyme discovered this year, menadione reductase (180) present in many plant and animal tissues, has been found in cell-free extracts of *Streptococcus faecalis* to require FMN or FAD (FMN is more active) (181) and to be FMN dependent in *Achromobacter fischeri* (182).

Mechanism of action, metallo-flavoproteins and electron transport.—A number of riboflavin enzymes have been recently shown to contain metals (metalloflavoproteins) (183, 184, 185) as listed in Table I (171).

TABLE I

METALS IN METALLOFLAVOPROTEINS

Metal		Enzyme
Copper	Cu^{++}	butyryl CoA dehydrogenase
Iron	Fe^{+++}	DPNH—cytochrome reductase
Molybdenum	Mo^{+VI}	xanthine oxidase aldehyde oxidase nitrate reductase hydrogenase

The copper-free enzyme prepared from butyryl CoA dehydrogenase (the green enzyme) by dialysis against KCN buffer can still be reduced by butyryl CoA[1] (as shown by changes in the flavin region of the spectrum) and is still capable of catalyzing the 2-electron transfer between butyryl CoA and dyes such as 2,6-dichlorophenolindophenol. However, this cyanide-treated enzyme cannot mediate such 1-electron transfers as the reduction of ferricytochrome-*c* or ferricyanide. This latter activity can be restored by adding $CuSO_4$. These findings indicate an intermolecular oxido-reduction from FAD to Cu and that Cu is required to transfer electrons from flavin to iron as shown in Figure 12 (171). With DPNH-cytochrome reductase, a ferroflavoprotein, the metal (Fe) has been shown to be involved in an intermolecular oxido-reduction and thus is required in the over-all transfer of electrons from flavin of the ferroflavoprotein to the Fe^{+++} of cytochrome-*c* (184). Xanthine oxidase similarly requires molybdenum for activity with cytochrome-*c*, but not for activity with dyes and oxygen, thus conforming to the pattern set by the other metalloflavoproteins (183).

Aldehyde oxidase of liver is another molybdoflavoprotein in which, as before, the metal is required for reduction of cytochrome-*c*, but not of dyes or O_2 (185). The enzyme, hydrogenase (from the obligate anaerobe *Clostri-*

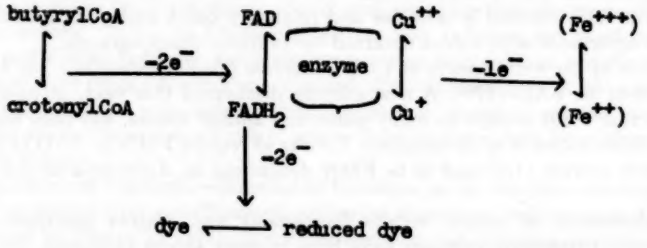
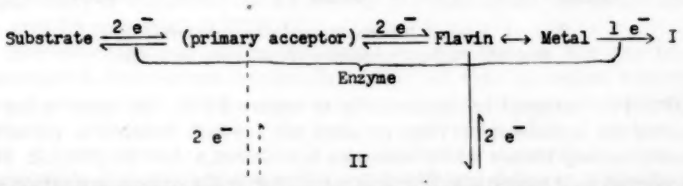


FIG. 12. Oxidoreduction of butyryl CoA dehydrogenase.

dium pasteurianum), which catalyzes the oxidation of hydrogen has been found to be a molybdoflavoprotein (186). Like the other enzymes in this series, the presence of Mo is required only for the oxidation of hydrogen by 1 electron acceptors like cytochrome-*c*, but not for oxidation of hydrogen by methylene blue (2 e^- acceptor). Mo has also been found to be a part of the flavin-enzyme, nitrate reductase of *Neurospora crassa* (187) and to play a similar role. In view of these findings, a generalization of the scheme in Figure 12 can be given (Figure 13) (171) in which the metal is involved in catalyzing the interaction of a 2-electron donor with a 1-electron acceptor.

The molar ratio of flavin-coenzyme to metal found was Cu:fl, 2:1 for the green enzyme; Fe:fl, 4:1 for DPNH[1] cytochrome reductase (however,



Acceptors of Class I	Acceptors of Class II
Cytochrome c Enzyme-bound cytochrome (Aldehyde Oxidase, Yeast Lactic Oxidase (?) Nitrate Ferricyanide	O_2 Redox dyes Quinones

FIG. 13. Mode of action of metalloflavoproteins.

in this case only 2 moles of Fe are in the Fe^{+++} state at optimum oxidation with cytochrome-*c*); and Mo:fl, 1:1 for xanthine oxidase. These are ratios which would in all cases permit electron transfer from the flavin to the metal component one at a time and might involve either the flavin-free radical concept (188) for the flavin semiquinone intermediary (Figure 14). It is suggested then that the special catalytic properties of metalloflavoproteins are related to this capacity of increasing the effective resonance of these active intermediates (flavin semiquinone, etc.) (189) as illustrated in Figure 15 (171, 190).

A similar line of thought is being followed in the study of a postulated role of riboflavin as a photocatalyst and electron carrier in photochemical reduction (191). In the presence of certain metal-chelating agents riboflavin was reversibly reduced by light, and this reduced riboflavin was able to tranfer its electrons to suitable hydrogen acceptors. Polarographic data again strongly suggest semiquinone formation. The evidence points to a metal-mediated complex between riboflavin and the metal-chelating agent as suggested above (Figure 15).

Microbial metabolism.—Strehler (192) has reported that the function of

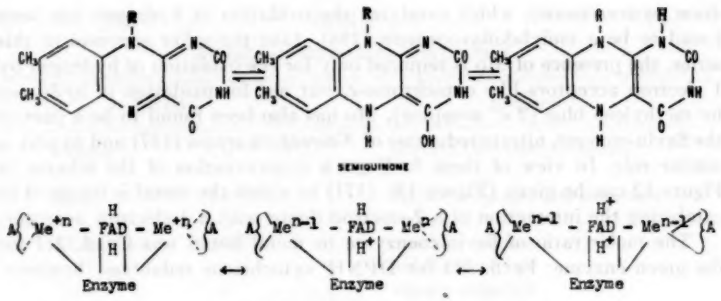


FIG. 14. (above) Riboflavin semiquinone intermediary.
FIG. 15. (below) Resonance stabilization by metal.

DPNH in bacterial luminescence is to reduce FMN, the latter being required for luminescence. Palmitic aldehyde has been isolated as the active factor in hog kidney which increases luminescence 100- to 200-fold. It is suggested that palmitic aldehyde is a cofactor in the oxygen activation system (193). A review on bioluminescence has appeared (194). A pteridine compound isolated from the developing grasshopper egg can replace riboflavin in the nutrition of a number of lactic acid bacteria including *Lactobacillus delbrueckii* etc. (195). Since the "pteridine" has an infrared absorption spectra similar to vitamin B_{12}, this would possibly indicate a benzimidazole nucleus which may serve as a precursor of riboflavin.

D-Riboflavin-2-C^{14} has been synthesized and its metabolism studied in *L. casei* (196). It was found that *L. casei* converts assimilated riboflavin to FMN and FAD at a ratio of about 1:16 with no free riboflavin left in the cells. In addition, an unidentified metabolite with an R_F of 0.78 (butanol acetic acid, water solvent system) was found which was not identical with the quinoxaline breakdown products obtained by alkaline degradation of riboflavin (see above). Only a small (0.2 per cent) part of the C^{14} was converted to CO_2.

Animal nutrition.—When weanling male mice were placed on a riboflavin-free diet, liver catalase levels fell in both riboflavin-deficient and pair-fed controls receiving riboflavin (197), the decrease being attributable presumably to the lowered food intake. However, if the mice were injected with testosterone after 4 to 5 weeks on the diet, a rise in liver catalase to normal adult levels was obtained in the positive pair-fed controls, but not in the riboflavin-deficient group. Riboflavin by injection is reported also to increase blood catalase in the human (198).

The relationship of riboflavin to protein and to fat metabolism in the rat has been re-examined using the antivitamin galactoflavin to produce a more rapid development of the deficiency. The results indicate that the utilizations of protein and of riboflavin are mutually limiting, for example,

on a low riboflavin diet the rat cannot use high protein levels (in fact, they appear toxic) and vice versa. Fat-free diets delayed the development of deficiency symptoms on galactoflavin; however, the mechanism is obscure (199).

In the study of embryonic malformations of dietary origin in rats (200, 201) omission of riboflavin from the diet of the dam has resulted in skeletal abnormalities in the off-spring, and it is suggested that this is a result of the lowered alkaline phosphatase and decreased rate of deposition of the bones.

Luecke and co-workers (202) have determined the riboflavin requirement of the baby pig to be approximately 3 mg./kg. of dry ration, which is about two times the requirement found for the weanling pig (203) and close to the requirement of approximately 2.5 mg./kg. of ration which was determined for sows for reproduction (204).

The riboflavin requirement for chicks for reproduction has also been investigated. For normal hatchability (205) 3.5 mg./kg. ration were required and analyses of the eggs showed that the minimum concentration necessary for normal hatchability was approximately 2.5 to 3 μg./gm. of yolk. A very interesting observation was the finding of a strain of chickens which, on a normal riboflavin intake, produced eggs deficient in riboflavin (206) so that all embryos died. Injection of riboflavin into eggs from these hens permitted hatching of normal chicks. The ration fed the hens contained 3.2 mg. riboflavin/kg. and treatment with increased amounts of riboflavin did not help. Evidently these hens were unable to transfer dietary riboflavin into the egg; however, the enzyme system blocked by this genetic variant was not determined.

Human nutrition.—In exophthalmic goiter (Basedow's disease) there is very little riboflavin excreted in the urine. When riboflavin is given (orally or by injection) it is very rapidly excreted and the urine again, after 8 hr., falls to the previous low level (207). The high excretions in this case apparently do not indicate saturated body stores, but metabolic failure in riboflavin utilization. Work on the riboflavin requirement of women during pregnancy indicates about 2.0 mg./day based on the intake required to bring the urinary excretion in pregnancy up to that of the nonpregnant woman (208). In pregnancy the placenta maintains the high (4×maternal fetal blood) concentration of riboflavin by splitting FAD built from maternal blood riboflavin (209).

Interrelationships. (*a*) With hormones: Normal rats and pair-fed controls of riboflavin-deficient rats subjected to a lowered oxygen tension equivalent to 20,000 ft. of altitude for 24 hr. exhibited glyconeogenesis; however, riboflavin-deficient rats were unable to respond. The administration of either adrenal cortical extract, or cortisone, or riboflavin previous to the anoxia test restored the glyconeogenic function completely. The role of cortisone in permitting, in riboflavin deficiency, the glyconeogenesis resulting from anoxia appears to establish adrenocortical failure as a part of the riboflavin deficiency syndrome. Since riboflavin administration immediately

repairs the defect, the damage must not be in the gland per se, and it is suggested that the impediment caused by the deficiency may lie in the stimulus of the pituitary-adrenal cycle (210). The effect of riboflavin deficiency on carbohydrate production is also seen in the markedly increased insulin sensitivity of the riboflavin-deficient rat (211) which frequently results in a fatal hypoglycemia.

(*b*) With carcinogens: Maximum excretion of free and conjugated 2-aminofluorene was obtained in dogs when the protein, riboflavin, and pantothenic acid contents of the diet were above the amounts needed for maintenance (212). In the rat a study of the metabolic fate of 2-acetyl-1-aminofluorene-9-C^{14} (213) has shown that riboflavin-deficient animals excreted a larger proportion of water-soluble metabolites such as the glucuronides of the hydroxylated compound and a smaller amount of ether-soluble compounds such as 2-aminofluorine, 2-acetyl aminofluorine, and their monohydroxy derivatives. However, no differences were found in the C^{14} content of the tissues.

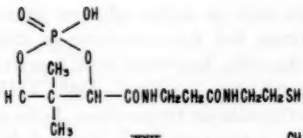
Pantothenic Acid

Because the functional form of pantothenic acid in the body is coenzyme A, this section of the review will cover together PA[1], CoA, and intermediate compounds.

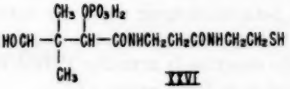
Chemistry.—An interesting, new, and practical method of synthesizing salts of PA has been devised which does not require the fusion of a metal salt of β-alanine with pantolactone. When equimolar amounts of pantolactone, β-alanine, and a secondary (or tertiary) amine are heated in anhydrous alcohol, a homogenous solution results from which calcium pantothenate can be obtained in high (88 per cent) yield on addition of calcium oxide (or sodium pantothenate by addition of sodium ethoxide) (214). Crystalline pantothenamide has been prepared from β-alanine amide and *dl*-pantolactone (215).

In a continuation of their extensive work on CoA, Baddiley *et al.* (216) have synthesized pantetheine-2′,4′-phosphate, XXV, and its disfulfide by (*a*) direct phosphorylation of pantetheine with phosphoryl chloride in moist pyridine, which gives the cyclicphosphate directly or (*b*) heating pantolactone-2-diphenylphosphate with an excess of the 2-mercaptoethylamide of β-alanine. Pantetheine-2′-phosphate, XXVI, was prepared by heating together pantolactone-2′-phosphate and the mercaptoethylamide of β-alanine (216). Synthesis of D(+)-pantetheine-4′-phosphate, XXVII, by direct phosphorylation of pantetheine with dibenzylphosphorylchloride was followed by removal of benzyl groups with Na in liquid NH_3 (217). Pantothenyl cysteine, XXVIII, has also been synthesized (218) by several different routes. These compounds were active as CoA precursors for *A. suboxydans* (219) but not for *Lactobacillus arabinosus.*

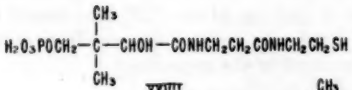
New syntheses of pantethine have been described (220, 221) involving



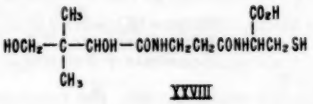
XXV



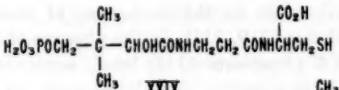
XXVI



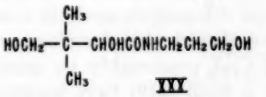
XXVII



XXVIII



XXIX



XXX

the condensation of D(−)pantolactone with di-(2-N-β-alanylamidoethyl) disulfide.

Biosynthesis of CoA.—A schematic representation of the total biosynthesis of CoA from the precursors, α-ketoisovaleric acid, aspartic acid, cysteine, and ATP, is given in Figure 16. Only the steps from F through J take place in mammalian tissue, the preceding steps occurring in microorganisms. These steps will be considered in turn, giving primarily the evidence published this year. Using *Bacterium linens*, an organism which requires either PA (or pantoic acid) and PABA[1] for growth, Purko, Nelson & Wood (222, 223, 224) have obtained evidence favoring the operation of pathway A-B-C (Figure 16) proposed by Kuhn & Wieland (225) and Maas & Vogel (226) and have demonstrated for the first time the biochemical hydroxymethylation of α-ketoisovaleric acid to give α-keto-β-β-dimethyl-γ-hydroxybutyric acid (ketopantoic acid). PABA was found to be required by *B. linens* (222) for this hydroxymethylation and was not replaceable by folic acid or leucovorin (except at very high levels). The same workers have also studied this hydroxymethylation reaction with enzymes from *E. coli* which catalyze the condensation of HCHO and α-ketoisovalerate to form α-keto-

pantoate (224). Possible C_1 donors such as serine, glycine, glucose, methionine, or formate did not substitute for formaldehyde in this reaction. Fractionated enzyme extracts of the cells, however, were inactive in carrying out the 1-carbon addition until supplemented with THFA,[1] metal ions^{++}, glutathione, and either methionine or tryptophan. Folic acid, leucovorin, and *p*-aminobenzoic were not activators. This would make it appear that, even though the nutritional requirement of *B. linens* for this reaction is PABA, the reaction is actually THFA catalyzed, as in the hydroxymethylation of glycine to form serine (227).

Reaction D is known from work on pyridoxine (co-decarboxylase) (228). Reaction E has been studied in *E. coli* by Maas (229) who found that the formation of this peptide bond involves a series of intermediary compounds containing the enzyme, as represented in the sequence:

$$\text{Enzyme (E)} + \text{ATP} \rightleftharpoons \text{E-PP} + \text{AMP} \quad 1.$$
$$\text{E-PP} + \text{pantoate} \rightleftharpoons \text{E-pantoate} + \text{PP}i \quad 2.$$
$$\text{E-pantoate} + \beta\text{-alanine} \rightleftharpoons \text{pantothenate} + \text{E} \quad 3.$$

This sequence would explain the requirement for ATP and the production of AMP and PP*i*[1] reported earlier (230). The requirement of cysteine for CoA synthesis (step F) is attributable to the formation of pantothenyl cysteine as a precursor of pantetheine (219, 231). In the absence of cysteine, PA is converted to pantothenyl 4′-phosphate (232) by *L. arabinosus* (step K). While these compounds are both inactive for *L. arabinosus* (as is CoA), they are both active for *A. suboxydans* and could, therefore, both be possible precursors of CoA presumably by parallel paths but different sequences (218). Brown & Snell (219) have suggested that phosphorylation of pantothenyl cysteine might occur prior to decarboxylation. Baddiley *et al.* (218) synthesized pantothenyl cysteine-4′-phosphate, XXIX, and found that it had only 40 per cent of the activity of pantothenyl cysteine for *A. suboxydans*, which might indicate that decarboxylation (step G) comes ahead of phosphorylation. Evidence from the animal side yields the synthetic pathway F through J from pantothenic acid to CoA (233). Supernatant fractions of rat liver gave with cysteine (specific requirement, not replaceable by any other compound) and PA the condensation to pantothenyl cysteine in the presence of ATP (step F). The pantothenyl cysteine was decarboxylated to pantetheine (step G) also by an enzyme in the rat liver supernatant fraction. The enzyme catalyzing the phosphorylation of pantetheine by ATP to yield 4′-phosphopantetheine (step H) has been partially purified from pigeon liver supernatant (234). The enzymes for steps I and J are in both mitochondrial and supernatant fractions and have been purified 100-fold from hog liver. Degradations yielding some of the same intermediates enzymatically from CoA show that at least some of these synthetic reactions are reversible (233, 235, 236).

The final structure of CoA in which the monophosphate grouping is attached at the 3 position of the adenosine moiety (reaction J) has been established by the use of bond-specific enzymes (237).

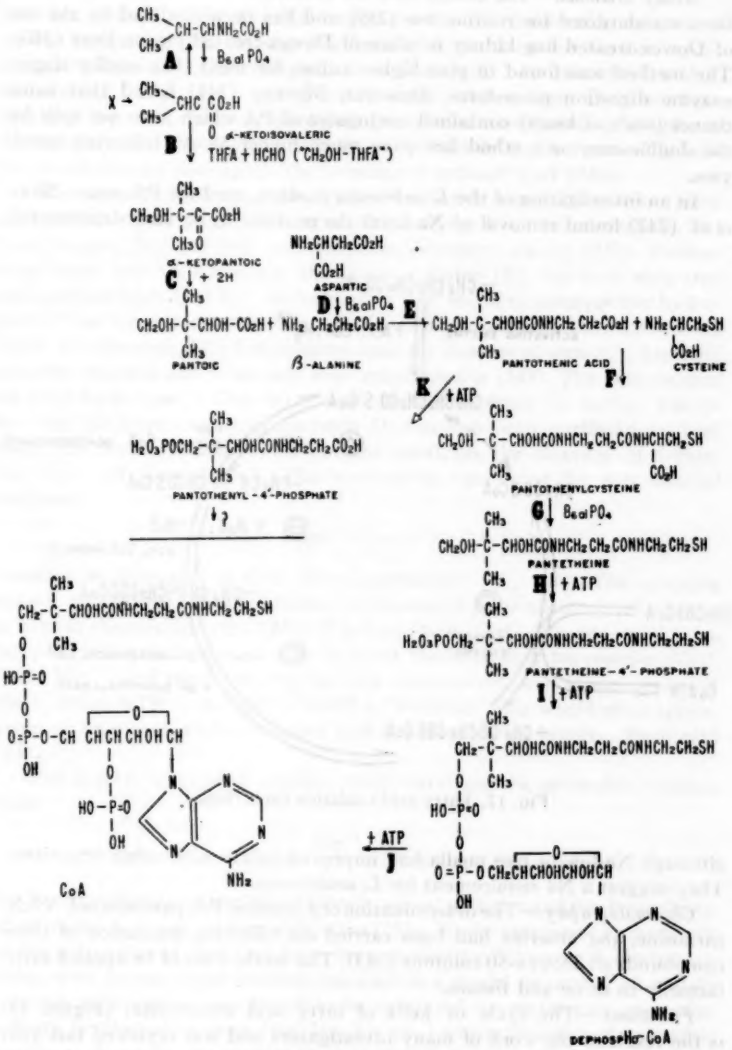


FIG. 16. Biosynthesis of coenzyme A.

Assay Methods.—The double enzyme method of Novelli *et al.* (238) has been standardized for routine use (239) and has been modified by the use of Dowex-treated hog kidney in place of Dowex-treated pigeon liver (240). The method was found to give higher values for meat than earlier single-enzyme digestion procedures. However, Marnay (241) found that some tissues (such as heart) contained conjugates of PA which were not split by the double-enzyme method but gave much higher assays following autolyses.

In an investigation of the *L. arbinosus* medium used for PA assay, Sirny *et al.* (242) found removal of Na from the medium to be very detrimental,

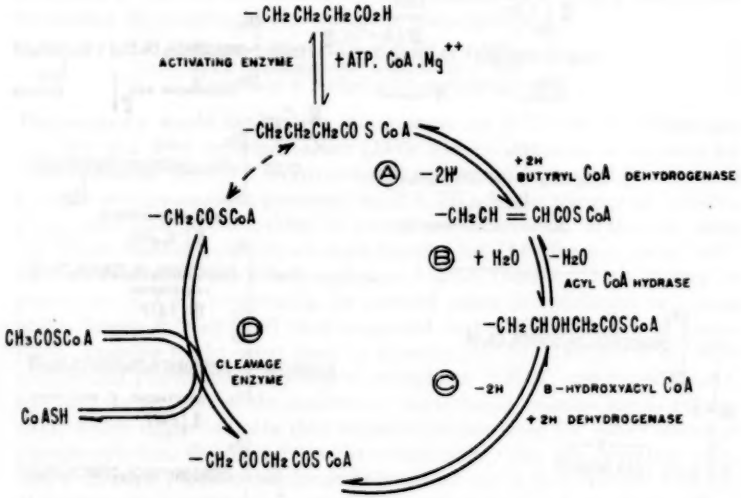


FIG. 17. Fatty acid oxidation cycle (helix).

although Na-low or free media had improved assays with other organisms. They suggest a Na requirement for *L. arabinosus.*

Chromatography.—The determination of β-alanine, PA, pantothenol, XXX, carnosine, and anserine had been carried out following separation of these compounds on Dowex-50 columns (243). The method could be applied satisfactorily to urine and tissues.

Functions.—The cycle or helix of fatty acid metabolism (Figure 17) is the result of the work of many investigators and was reviewed last year (244, 245). Recent work has centered on the four enzymes involved in the process, their coenzymes, and their specific substrate requirements. The overall reaction can be written:

$$CH_3(CH_2)_nCOSCoA + CoASH \rightarrow AcCoA + CH_3(CH_2)_{n-3}COSCoA$$

and the AcCoA is the 2-carbon substrate for the condensing enzyme of citrate formation, thus linking this helix with the tricarboxylic acid cycle. Various β-keto acid CoA compounds (acetoacetyl, β-ketohexanoyl, and β-ketoctanoyl CoA), substrates of the cleavage enzyme (step D), have been prepared by enzymatic dehydrogenation of the corresponding β-hydroxy derivatives (step C). The compounds are chemically stable but readily subject to enzymatic cleavage in the presence of reduced CoA (246).

The enzyme which catalyses the first oxidative step (A) for lower fatty acids (C_3 to C_8-optimum activity at C_4), butyryl CoA dehydrogenase, has been isolated and purified (see riboflavin functions above) (247). Unsaturated fatty acyl CoA hydrase, the enzyme of step (B), has been separated and purified from beef liver mitochondria and found to catalyze the hydration of the *trans*-isomers of α,β and β,γ-unsaturated acyl CoA derivatives (248). β-hydroxyacyl CoA dehydrogenase, the enzyme of step (C), has been partially purified also from beef liver mitochondria (249). The enzyme acts on all β-hydroxyacyl CoA fatty acid derivatives from C_4 to C_{12}. The β-ketoacyl CoA cleavage enzyme (step D) has also been purified from beef liver mitochondria (250). This enzyme catalyzes the cleavage of β-keto, C_4-, C_6-, and C_8-acyl CoA. The mechanism postulated for this over-all reaction:

$$AcAcCoA + \text{enzyme} \rightleftharpoons \text{enzyme} + 2Ac\ CoA$$

assumes the formation of E-Ac as an intermediate (cf p. 444). The complete helix has been reconstructed from its component parts with triphenyltetrazolium as electron acceptor (251). The fatty acid (C_4–C_{18}) is converted to its fatty acyl CoA derivative in order to enter the helix. For this reaction ATP, CoA, Mg^{++}, and the activating enzyme are required, and in the course of the reaction ATP is cleaved to AMP+PP*i* (252). This would thus appear again to be a several-step reaction and apparently also involves lipoic acid (253).

The acetate activating enzyme which catalyses the reversible reaction (254):

$$ATP + CoA + \text{acetate} \overset{Mg^{++}}{\rightleftharpoons} AMP + \text{acetyl CoA} + PPi$$

has been purified from beef heart mitochondrial particles and has been shown to be unstable protein after purification (255). Model reactions for the study of the activation of acyl CoA have been studied by Schwyzer & Hürlimann (256). (+) S-benzoylpantetheine was used as a model for acyl CoA and its reaction with aniline was found to be catalyzed by metal ions possessing affinity for the sulfur atom (Ag, Hg, Pb, etc.) which thus served as models for the acylases. On the basis of these models, it was postulated that the acyl transfer is attributable to the electrophilic character of CoA.

Phospholipide synthesis has been found to be CoA and respiration de-

pendent (257, 258), and this is interpreted as indicating that the fatty acid ester bonds of phospholipides are formed by transesterification from acyl CoA.

In the metabolism of the amino acid leucine, the role of CoA in the breakdown of the isovaleryl carbon chain by thiol-ester formation has also recently been demonstrated with the interesting new finding of CO_2 fixation by CoA activated β-hydroxy-β-methyl glutaric acid according to the reaction sequence:

$$\text{IsovalerylCoA} \rightarrow \text{senecioylCoA (the } \alpha, \beta \text{ unsaturated thiol ester)} \quad 1.$$

$$\text{SenecioylCoA} + H_2O \rightarrow \beta\text{-hydroxyisovalerylCoA} \quad 2.$$

$$\beta\text{-hydroxyisovalerylCoA} + CO_2 \xrightarrow{\text{ATP}} \beta\text{-hydroxy-}\beta\text{-methylglutarylCoA} \quad 3.$$

$$\beta\text{-hydroxy-}\beta\text{-methylglutarylCoA} \xrightarrow[\text{or GSH}]{\text{cysteine}} \text{acetoacetate} + \text{acetylCoA} \quad 4.$$

The first two reactions are analogous to those of the fatty acid cycle (see above) and, along with the second two, have been demonstrated individually in beef heart extracts (259).

Cholesterol synthesis.—The role of CoA in the formation of "active acetate" (acetyl CoA) (260) and the role of acetate as the primary precursor of sterol synthesis in the animal body has led to an investigation of the effects of PA and CoA on cholesterol synthesis by a number of investigators. In a series of rat feeding experiments Boyd (261) has shown that a decrease in plasma and liver cholesterol (particularly cholesterol ester) does, in fact, occur as a result of dietary PA deficiency. *In vitro* work with rat liver "homogenates" has demonstrated that CoA is an activating factor for cholesterol synthesis (262) and the following pathway has been proposed (263):

$$CH_3COCH_2CO_2H + CH_3CO_2H \rightarrow CH_3\text{—}\underset{\displaystyle CH_2CO_2H}{\overset{\displaystyle OH}{\underset{|}{\overset{|}{C}}}}\text{—}CH_2\text{—}CO_2H \xrightarrow{-H_2O} CH_3C{=}CHCO_2H \quad (CH_2CO_2H \text{ on the } C)$$

The intermediate β-hydroxy-β-methyl glutaryl CoA has been previously demonstrated (259) and this pathway undoubtedly involves CoA at all steps. C^{14} labeling of these postulated intermediates showed their incorporation into cholesterol to be equal to that of acetate.

PA and CoA in microbial metabolism.—*A. suboxydans* has been one of the most useful organisms for investigations of the role of pantothenic acid because of its optimum response to CoA and its response to the intermediates in CoA synthesis from pantoic acid up. Two investigations (264, 265) of the effectiveness of various PA conjugates on the growth of this organism have shown that the —SH compounds, but not the —S—S— compounds, are active. Under optimal conditions (i.e., presence of reducing agents) CoA, 4′-phosphopantetheine, pantetheine, and pantothenyl cysteine show similar activities for *A. suboxydans*, activities that are several times those of PA or pantoic acid. One of the spirochetes, *Treponema pallidum* S-69 has been shown to

require CoA, and this requirement is replaceable by 4'-phosphopantetheine, while earlier precursors of CoA were inert (266).

The decarboxylation of succinyl-CoA by *M. lactilyticus* has been described as yielding propionyl CoA+CO_2 (267). On the other hand, precipitates of homogenates of *Tetrahymena pyriformis* convert succinyl CoA directly to acetyl CoA (268). ATP and Mg^{++} are required for the reaction, and no intermediates (propionyl CoA, etc.) nor CO_2 appear to be produced in the reaction.

In the case of *Clostridium saccharobutyricum* (269) the acetyl CoA formed by the oxidative decarboxylation of pyruvate results in the formation from the required ATP of orthophosphate, rather than pyrophosphate (254) as in animal systems, as a result of the transfer of the acetyl to acetyl phosphate by phosphotransacetylase. In the organism *Pseudomonas fluorescens* CoA is involved in the decarboxylation of malonate (270) in a series of reactions which would appear to be analogous to those for the decarboxylation of succinyl CoA (259).

Animal nutrition.—Pantethine and pantetheine, whether given orally or by injection, were found equal to PA in vitamin activity for the chick (271). However, pantothenyl cysteine was without vitamin activity for both rats and chicks whether given orally or by injection (272), a result which appears quite unexpected in view of the role of pantothenyl cysteine as an intermediate between PA and pantetheine, both of which were active. Also surprising was the report that pantoyltaurine and PA were equally effective in protecting rats against P^{32} irradiation damage. The mechanism of action, however, is not understood and may not depend on vitamin activity (273).

The effect of PA deprivation during pregnancy has been studied by two groups of investigators. It was found that in severe PA deficiency there was complete litter resorption or abortion (274, 275), while in milder deficiency states (intakes of 20 to 30 μg. PA/day/rat) there occurred fetal abnormalities of various types (276). When the maternal PA-free diet was supplemented with 2 per cent ascorbic acid, the effects of the deficiency were alleviated (277). The PA concentrations in the tissues of the young at birth and of the dam were higher when the ascorbic acid was included in the diet, and the blood PA values were comparable to those of stock young; while without ascorbic acid on the PA-free diet, the young possessed lower PA concentrations.

The PA requirements of the pig have been studied at different periods in the life of the animal. Luecke (278) found the requirement for the baby pig (preweaning age) to be 10 to 15 mg. PA/kg. diet (at 10 mg. one pig showed the typical locomotor incoordination of PA deficiency). For the growing pig (60 to 160 days of age) Moustgaard (279) has reported a requirement of 400+μg. PA/kg. body wt./day for optimum nitrogen retention, although a level of 200 μg. was adequate for growth. A level of approximately 10 mg./kg. diet has been found necessary for the sow for reproduction (280).

In work on the relation of nutrition to infection it has been found that

PA-deficient rats are susceptible to infection by a corynebacterium pathogenic to mice (281) and to a "pseudotuberculous" organism (282). Neither organism is pathogenic to rats receiving adequate PA, while the break in resistance can be demonstrated by inoculation of PA-deficient rats before any apparent deterioration in physical condition attributable to the deficiency has developed.

Human nutrition.—Using the antivitamin, ω-methyl pantothenic acid, which inhibits lactic acid bacteria competitively with PA (283), Bean & Hodges (284) have been able to bring about a PA deficiency state in four human volunteers. The chief symptoms were those of adrenal insufficiency and peripheral neuropathy. The adrenal symptoms again point to the importance of CoA in the synthesis of sterol for adrenosteroid production.

Interrelationships.—(*a*) Antibiotics: PA deficiency can be alleviated not only by ascorbic acid (277) but also by antibiotics. When antibiotics are fed the growth of rats (136) and poults (285) on low PA diets is improved, and in addition it has been found that the deficit in antibody formation which results from PA deficiency in the rat no longer occurs (286).

(*b*) Hormones: A relationship of PA to the adrenal gland has long been recognized. The most obvious reason for such a relationship is the role of CoA in acetate activation and thus in cholesterol synthesis, the cholesterol then serving as substrate for adrenosteroid formation. Dumm & Ralli (287) have found, in fact, that the PA-deficient rat is unable to resynthesize adrenal cholesterol following the stress of unilateral adrenalectomy. The stress condition of anoxic anoxia has also been used to study the effect of PA on adrenal function (288). Under nonstress conditions PA-deficient rats had lower adrenal cholesterol, liver glycogen, and blood sugar levels and higher adrenal weights and adrenal ascorbic acid levels than nondeficient rats. After exposure to 20,000 ft. equivalent altitude for 24 hr. without food or water, the nondeficient rats showed a 15-fold increase in liver glycogen and a significant rise in blood sugar as compared to nonstress fasted controls; while the PA-deficient rats, on the other hand, showed no change in either liver glycogen or blood sugar. If adrenal cortical extract (288) or cortisone (289) was given before the stress, the PA-deficient rats showed a normal carbohydrate response; yet adrenal cholesterol had fallen in both groups following stress. It thus appears that the level of adrenal cholesterol per se is not an index of the amount of adrenosteroid hormones which can be produced. This may possibly indicate a role of CoA in the hormone synthesis from sterol.

myo-INOSITOL

Assay.—An assay method for the quantitative determination of lipide inositol in animal tissues (290) is an adaptation of the *Saccharomycetes carlsbergensis* procedure (291) in which the ester linkages are hydrolyzed completely by a 40 hr. hydrolysis with 4 *N* HCl. By this procedure lipide inositol was found in all tissues analyzed, and it appears that in the plasma, at least, lipide inositol accounts for the major part of the total inositol.

Inositol phospholipides.—The inositol phosphatides of soybean and ox-brain have been prepared and analyzed. In the case of the inositol lipides from brain, paper chromatography following hydrolysis with 6 *N* NCl for 25 min. at 100°C. showed three phosphoric ester areas, two of which were identifiable as inositol diphosphate and inositol monophosphate. Soybean phosphatide included, in addition, compounds thought to be the galactoside and arabinoside of inositol monophosphate (292). The inositol-containing phosphatides (including inositol diphosphate) of soybean can be separated from the N-containing phosphatides by lead precipitation methods (293). A monophosphoinositide has been obtained intact in rather pure form from liver (294). The preparation contained two molecules of fatty acid per atom of phosphorus.

Free inositol.—In contrast to the occurrence of inositol almost entirely in the bound form in animal tissues, Mann (295) has found large quantities of free inositol in seminal fluid of the boar. Free inositol was present at a level of approximately 20 per cent of the dry weight or 40 to 70 per cent of dialyzable dry weight of seminal fluid. No function of this inositol could be found in relation to sperm cell metabolism, and it is speculated that it may function in the maintenance of the osmotic pressure of the fluid. This work might suggest the pig as an experimental animal in which to attempt to produce an inositol deficiency. However, early work with the baby pig demonstrated no effect on the deficiency and only a slight lipotropic action of the compound when fed on a choline-free diet (296). Likewise, in recent work with rats fed a choline-free, amino acid synthetic diet, no growth differences and no pathology were found in the absence of dietary inositol over a 16-week period (297). The urinary and fecal excretion of inositol was not lowered by its omission from the diet, nor was the inositol content of the carcass decreased. These experiments would support the thesis that the body tissues are able to carry out the synthesis of inositol.

Function.—Whether from an endogenous or exogenous source, it seems evident that inositol is required by the body. The function most commonly assumed for inositol is that of fat transport as inositol-phospholipide and many investigators have claimed lipotropic function for inositol. In recent work Cornatzer *et al.* (298) have found no effect of added inositol on phospholipide synthesis and a slight lipotropic effect only in rats on a low-fat diet.

Hartman (299) has suggested that inositol functions in protein metabolism, specifically in the liberation of methionine from protein, thus accounting for any lipotropic effect.

Inositol has also been claimed to play a role in α-amylase activity. Rats fed a diet containing the anti-inositol compound, the γ-isomer of hexachlorocyclohexane, developed alopecia in 10 to 12 weeks, and the amylase activity of the blood plasma was lower than that of rats fed the same diet plus inositol (300).

Microbial metabolism: Function.—In the case of many microorganisms there is no doubt as to an exogenous requirement for inositol, and the fact that it thus must function in microorganisms plus its occurrence in animal

tissues is perhaps the best reason for postulating a function in animal tissues.

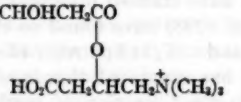
When cells of the yeasts *Kloeckera brevis* or *S. carlsbergensis* were deprived of inositol, rapid death of the majority of the population occurred accompanied by a loss of metabolic activities, nucleotide coenzymes, and the cytochrome system although free porphyrin synthesis was not diminished (301). It was postulated that inositol functions as a structural component of the yeast cell, and in support of this theory it was found that most of the cell inositol occurred in the cytoplasmic particles in bound form. Inositol seems to play a similar basic role in metabolism in *Neurospora* (302) since in the presence of the inositol antagonist isomytilitol, this organism shows a decreased ability to carry out the synthesis of B_2, B_6, NA, PABA, and PGA,[1] although not of PA or choline.

Metabolism.—The organism *Aerobacter aerogenes* adapted to inositol degrades it via 2-keto-*myo*-inositol and L-1,2-diketo-*myo*-inositol followed by cleavage of the cyclohexane ring into an equal mixture of a 3-carbon compound, a 2-carbon compound, and CO_2 (303).

Miscellaneous

Adenine.—Even though adenine is synthesized by body tissues, it continues to be regarded by some laboratories as a vitamin (B_4) involved in blood cell formation (304).

l-carnitine.—Carnitine $[(CH_3)_3\overset{+}{N}CH_2 \cdot CHOH \cdot CH_2 \cdot COO^-]$ a vitamin (B_t) required by the mealworm and some other beetle larvae, has been found to be very widely distributed in animal tissues (305). The carnitine content of muscle is, in general, higher than that of other tissues (e.g., chick muscle 175 μg./gm. dry weight; liver 20 μg./gm. dry weight). From work with germ-free chicks it is evident that carnitine is synthesized by the animal tissues, but its synthetic pathway has not been elucidated, nor is the function of carnitine in the body known. However, the esters of carnitine have an acetyl choline-like stimulating effect on isolated muscle (306). It is not a satisfactory methyl-donor for the rat (307). It can be replaced in the nutrition of *Tenebrio* by synthetic dicarnitine:



which is equally active (308). Carnitine can be determined chemically, following isolation by paper chromatography or paper electrophoresis, by conversion to crotonic acid betaine and titration with $KMnO_4$ (309).

Orotic acid.—A relationship has been suggested between the pyrimidine orotic acid and the postulated animal vitamin B_{13} (310). Both are active for rats, chicks (311), *Lactobacillus bulgaricus*, and other microorganisms, and the response obtained from whey or other milk products in chicks (311) and pigs (312) can be duplicated with orotic acid. However, concentrates of B_{13} from distillers' solubles are active at the microgram level as compared

to the milligram level for orotic acid and it is, therefore, suggested that orotic acid is only a part of the B_{12} molecule (310). On the other hand, the biosynthesis of orotic acid in rat-liver slices (313) and in pigeon liver slices (314) via the pathway, citrulline+aspartate→ureidosuccinate→dihydroorotic acid→orotic acid has been demonstrated, thus proving that orotic acid is not a vitamin for these species. It is claimed to be dietary essential for mice (315), a plateau in weight occurring after 11 days in its absence, while normal growth was obtained with mice receiving 5 mg./day. Orotic acid is converted by the rat liver *in vivo* into uridine-5′-phosphate, which appears to serve as an immediate metabolic precursor of the uracil of ribonucleic acids (316) and of other important uridine compounds (such as uridine diphosphoglucose, etc.).

The requirement of certain microorganisms for orotic acid is, on the other hand, well-established (317, 318). Orotic acid is replaceable in the case of *L. bulgaricus* 09 by L-dihydroorotic acid (319), uridine-5′-phosphate, and cyclic uridine-2′,3′-phosphate (320). The orotic acid fermenting bacterium *Zymobacterium oroticum* catabolizes orotic acid (321) by the same pathway shown for its synthesis in the liver (314 above).

Stilbestrol.—Diethyl stilbestrol, which has long been known to give growth responses in various species of livestock when implanted as pellets subcutaneously, has recently been shown to improve markedly gains of steers when fed at low levels in the diet (322). The diethyl stilbestrol has been fed to steers at levels of 2.5 to 10 mg./day and to lambs at 0.6 to 1.2 mg./ day, levels (323) in both cases promoting increased gains of 20 to 30 per cent over the basal ration as well as improving feed efficiency.

Strepogenin.—Strepogenin activity (a growth factor activity for bacteria) formed during partial hydrolysis of certain proteins, has been found to reside in several peptides which were purified by ion-exchange chromatography (324). The amino acid composition of the purified peptides was observed to be very similar to that of the hormone, oxytocin, and on testing it was found that synthetic oxytocin did, in fact, possess high strepogenin activity. The fact that the synthetic peptide is highly active proves that the activity obtained from natural materials is attributable to the activity of the peptides per se and not to some contaminant. While considerable specificity of structure is involved in strepogenin activity, a number of different peptides do possess activity.

Bifidus factor.—The growth factor for *Lactobacillus bifidus* var. Penn., present in human milk but not in cow's milk, has been identified as galactosido-N-acetyl-D-glucosamine (325, 326). In addition the synthetic compound, β-methyl-N-acetyl-D-glucosamine, was found to be active for this organism (327). Evidence has been presented that crude preparations of the *L. bifidus* factor are active in promoting the growth of rats on a dilute cows' milk ration (328, 329).

An unidentified growth factor for an avian *L. bifidus* T_3 has been described (330) and found to be a different factor. This organism does not respond to galatosido-acetyl glucosamine. The factor is present in yeast and

bacterial fermentation products, in beef extract, and in fish solubles. It is soluble in water and methanol, but not in ether or absolute ethanol, and is adsorbed on charcoal (Norite).

Other microbial factors.—During the year several new bacterial factors have been proposed, as well as some earlier ones identified. Many sugar derivatives of glycine such as N-D-glucosyl glycine are active for *Lactobacillus gayoni* (331), perhaps as precursors of a naturally occurring *L. gayoni* factor. Other lactic acid bacteria factors include: lactein (apparently a protein), an absolute requirement for growth of a strain of *L. bulgaricus* (332), and a new factor from natural sources for growth initiation and acceleration of *L. casei* (333) and for *S. faecalis* (334).

Copragen, the factor required by *Pilobolus kleinii* has been isolated in crystalline form (335) from *Penicillium* fermentation. It is an organic iron compound with an absorption maximum at 440 mμ and may be a porphyrin compound. It is active as is ferrichrome (336) as the "Terregens" factor required by *Arthrobacter pascens* (337). The flagellate *Tetramitus rostratus* has been found to require two unidentified factors present in liver and in *Bacillus cereus* or *B. subtilis* cells (338).

Unidentified factors appear to be required by rumen bacteria for growth and cellulose digestion (339 to 342). The active factor or factors are present in rumen fluid and also in yeast, forages, feces, etc.

Chick factors.—While a great deal of work continues to be published on unidentified factors for chick growth, little progress has been made on their concentration or identification. In general terms they can be separated into (*a*) the "fish factor" or liver factor present in fish meal, fish solubles, liver, etc. (343 to 347); (*b*) "whey factor" [which may be replaceable by orotic acid (311)] present in whey, butyl fermentation solubles, and dried brewer's yeast (343, 348, 349); (*c*) "alfalfa factor" (346), as well as factors which may or may not be identical in egg yolk (344), penicillin residues (350), a forage juice concentrate (351), and autoclaved litter (352). Turkey poults respond to an unidentified factor in yeast by increased growth and a decrease in hock disorder (353).

Rat and mouse factors.—An unidentified factor, factor 3, is involved along with cystine and vitamin E in the prevention of dietary liver necrosis in the rat. Some concentration of factor 3 has been obtained, and it has been found to be of low molecular weight, stable to acid hydrolysis, and water soluble (354).

A heat-stable factor in the forestomach and intestine of newborn rats has been found to possess a marked mitosis-stimulating effect on the stomach epithelium of 4 to 6 week old rats following intraperitoneal injection (355) while a plasma extract from rabbits treated with phenylhydrazine has proven hematopoietic when injected into normal rats. The nature of the factor was not determined (356).

The nutritional factor present in wheat which promotes resistance of the mouse to Salmonella infection has been highly concentrated from wheat midlings by methanol extraction and ion-exchange chromatography (357).

Swine factors.—While fish solubles have been reported to possess unidentified vitamins for swine and poultry (as noted above), it appears that in the case of pigs, at least, the effects may be attributable entirely to the supplemental effect of its protein content (358). An unidentified growth factor for the pig has been reported to be synthesized in the intestinal tract, probably by *Aspergillus flavus*. This new factor can be assayed by *S. faecalis* and is not present in yeast extract, corn steep liquor, or peptone (359).

Calf factor.—It appears possible that the growing calf requires, as yet, unidentified nutrients. When calves are raised on a mineralized whole milk ration only, they die at from 5 months to 1 year with symptoms which do not appear on a normal roughage plus grain-type ration (360).

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FAT-SOLUBLE VITAMINS[1,2]

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With the exception of the participation of vitamin A in vision, biochemistry has not yet achieved an understanding of the function of the fat-soluble vitamins on a molecular basis. Progress towards this difficult goal during the past year has been limited. Many reports have filled gaps in areas previously studied. In some instances the experimental evidence does not warrant conclusions or suggestions made. None the less, significant advances in the field have been made. Within the limitations of space and the competency of the reviewer, attention is directed to what are regarded as important contributions.

Vitamin A

Goodwin has discussed carotenoids elsewhere in this volume, and only those aspects directly related to vitamin A activity will be considered here.

Chemistry.—Progress in the synthesis of various isomers of vitamin A and related compounds in the past several years has been gratifying. Preliminary reports have appeared describing some excellent work on the preparation of geometrical isomers of vitamin A and vitamin A aldehyde [Robeson, Cawley *et al.* (1 to 4)]. The vitamin A isomers were prepared by condensation of β-ionylideneacetaldehyde with methyl-β-methylglutaconate, decarboxylation of the resulting 4-carboxyvitamin A acid, and reduction of an ester of the acid with lithium aluminum hydride. Experiments on the condensation of various aldehydes with methyl-β-methylglutaconate (3) and on the chemistry and stereospecificity of products obtained by decarboxylation of γ-arylidine-β-methylglutaconic acids gave information basic to the studies with vitamin A (4). The four isomers prepared were assigned configurations as all-*trans*, 2-*cis*, 6-*cis*, and 2,6-di-*cis*.[3] The corresponding geometrical isomers of vitamin A aldehyde were also prepared (2); preparation of the 2,6-di-*cis* isomer has not been previously reported. The important visual pigment neoretinene b was obtained in crystalline form after exposure of the all-*trans* aldehyde to light followed by chromatographic separation [Dieterle & Robeson (5)]. In confirmation of earlier results, configurational

[1] The literature survey was completed in November, 1954. The able assistance of Mrs. Madelyn Ferrigan in the preparation of this review is gratefully acknowledged. Only isolated aspects of the clinical uses of fat-soluble vitamins are in this review.

[2] The following abbreviations are used: ADP for adenosinediphosphate; ATP for adenosinetriphosphate.

[3] In the numbering system used the terminal alcoholic carbon of the side chain is assigned the number 1 as in last year's review by Quaife [*Ann. Rev. Biochem.*, **23**, 215 (1954)]; this differs from the system used by Wald [*Ann. Rev. Biochem.*, **22**, 497 (1953)].

studies indicate that neoretinene b is 2,4-di-*cis*-vitamin A aldehyde and thus has a so-called "forbidden *cis*" configuration (2).

Oroshnik (6) presents evidence that "isoanhydrovitamin A," obtained by the action of dilute ethanolic HCl on anhydrovitamin A is not a hydrocarbon but is actually an ethyl ether. Vitamin A adsorbed on sodium bentonite may be converted to anhydrovitamin A and other products (7). The synthesis and properties of α-vitamin A methyl ether have been reported in detail (8), and the chemistry and synthesis of vitamin A has been reviewed (9).

Important syntheses in the carotenoid series by Inhoffen and associates (10, 11, 12) include the preparation of all-*trans*-D,L-α-carotene using the principles successfully applied to β-carotene synthesis. For the synthesis a C_{19}-aldehyde was reacted with acetylene to give a C_{21}-hydroxyethinyl compound and the latter condensed with a C_{19}-aldehyde to give a C_{40}-diol. The diol was dehydrated, then reduced to give 15,15′-mono-*cis*-D,L-α-carotene, which, on treatment with iodine, gave all-*trans*-D,L,-α-carotene. The D,L product melted at 160.8–162.8° C. as compared with 187–188° C. for the natural, optically active α-carotene.

Biopotency of vitamin A isomers and related compounds.—Valuable basic information about the biological activity of geometric isomers of vitamin A and vitamin A aldehyde has been reported. Crystalline neoretinine b has only about ½ the biopotency of all-*trans* vitamin A aldehyde as measured by a rat liver-storage method (5); thus this bioassay method does not reflect the biological importance neoretinine b is considered to have in the formation of the visual pigment rhodopsin. The 2-mono-*cis* (neovitamin A), has about 75 per cent and the 6-mono-*cis*, 2,6-di-*cis*, and 2,4-di-*cis* isomeric vitamin A acetates have about 23 per cent of the activity of all-*trans* vitamin A acetate as measured by growth and liver storage bioassays [Ames, Swanson & Harris (13)]. In similar assays the all-*trans* and 2-mono-*cis* vitamin A aldehydes showed 91 per cent, the 2,4-di-*cis* about 47 per cent, and the 6-mono-*cis* and 2,6-di-*cis* about 18 per cent of the molar bioactivity of the all-*trans* vitamin A acetate. The higher activity of the 2-mono-*cis* and 2,4-di-*cis* aldehydes than the corresponding isomeric acetates suggests that an equilibrium is established *in vivo* between the 2-*cis* and 2-*trans* forms of the aldehydes but not between the acetates (14,15). The data also suggest that conversion of vitamin A to the aldehydes *in vivo* is limited.

Garbers, Eugster & Karrer (16) report that for rats *cis*-β-carotene C has about ¼ to ⅓ of the vitamin A activity of all-*trans* β-carotene; the greater instability of this isomer compared with other *cis*-β-carotenes is probably responsible for the reduced biological activity. Synthetic isocryptoxanthin (4-OH-β-carotene) has practically the same biopotency as naturally occurring cryptoxanthin (3-OH-β-carotene) (17).

Convincing evidence has appeared that the vitamin A activity of lard is attributable to the presence of vitamin A itself and not to some other factor with vitamin A activity (18, 19). Herb *et al.* (18) found that chromato-

graphic fractionation of unsaponifiables from lard and molecular distillates from lard yielded eluates which gave positive Carr-Price tests and typical vitamin A spectral curves. Even more convincing evidence has been supplied by Ames & Harris (19) who demonstrated the presence of vitamin A by application of the Carr-Price reaction and by application of the anhydrous HCl dehydration procedure to form anhydrovitamin A; difference spectra showed the characteristic triple-peak curve of anhydrovitamin A. The amounts of vitamin A present were sufficient to account for all of the biological activity. The inability of Lowe & Morton (20) to detect vitamin A in lard distillates appears to be principally attributable to lack of sensitivity of methods employed. The presence of vitamin A in lard probably accounts for the activity reported by Nieman (21). At present there is no good evidence that compounds other than vitamin A or very closely related substances possess vitamin A activity. The experiences with the "lard factor" point out the restraint that needs to be used in postulating the existence of unrecognized factors with biological activity.

Stability during storage.—Stabilization of carotene and vitamin A in feeds remains a problem. Walsh & Hauge (22) have attempted to evaluate the relative importance of enzymic and nonenzymic processes in carotene destruction; definitive results on this question prove difficult to obtain. The most effective of various antioxidants related to aniline was N-N′-diphenylhexamethylenediamine (23); the carotene retention was influenced by the amount of oil used in applying the antioxidant. Bickoff *et al.* report that of various quinoline derivatives tested, 6-OH-2,2,4-trimethyl-1,2-dehydroquinoline was the most effective for carotene in oil but was less effective than the corresponding 6-alkoxy derivative for carotene in alfalfa meal (24). The loss of vitamin A as cod liver oil was greater in coarse than in fine feed mixtures, was reduced by pelleting and, as noted by others, increased by mineral salts (25); the effect of mineral salts was reduced by gelatin. Siedler & Schweigert (26) found that dry dog feeds retained vitamin A as well or better when 6 per cent of antioxidant-treated fat was incorporated into the feed. They stress the feasibility and desirability of additions of stabilized fats to feeds. Other papers deal with the stability of vitamin A or carotene in milk (27), in ghee (28), and in vegetable and mineral oil (29), and during the processing and storage of peanut butter (30) and of peas and lima beans (31). An off-flavor in vitamin A fortified skim milk has been found to result from the action of light (32).

While the use of chemical stabilizers offers promise for improvement of carotene and vitamin A retention during storage, emphasis must be given to the possible toxicity of the added stabilizers until they have been proven innocuous for many species over long periods.

Assay procedures.—Reports have been limited to variations and applications of existing methods. Wilkie & Jones (33) recommend that alumina for chromatography in vitamin A assay be quantitatively evaluated. What appear to be adequate methods have been described for whale liver oil (34),

margarine (35, 36), and liver (37). The procedure for liver involves only grinding with sodium sulfate, mixing with ether, and performing the Carr-Price reaction on an aliquot of the extract; it is precise and reliable and has been used in the development of a satisfactory bioassay based on the amount of liver storage [Ames, Risley & Harris (37)]. Various methods for estimation of blood plasma vitamin A in calves have been compared statistically (38).

Bruggemann *et al.* (39) give an extensive theoretical treatment of the determination of vitamins A and D with electrophilic metal halides. Their considerations lead to a procedure of potential value (40) in which treatment with $FeCl_3$ converts vitamin A to polyenes with 5 to 6 double bonds. The addition of acetic anhydride solubilizes the $FeCl_3$ and yields a stable blue color. From studies on the rate of fading of the color of the Carr-Price reaction, Wagner *et al.* (41), conclude that interfering substances commonly encountered decrease the maximum color produced. McCoord (42) has pointed out advantages for 1,2-dichloroethane as a solvent for $SbCl_3$ in the Carr-Price reaction. Flesch (43) has described a color reaction of vitamin A and carotene which may be used for analytical work; inexpensive reagents, perchloric acid and amyl acetate, are used, but sensitivity is low and the color fades.

Interference of the antioxidant N-N′-diphenyl-*p*-phenylenediamine in carotene determination may be avoided by the use of tricalcium phosphate instead of MgO as an adsorbent [Mitchell & Silker (44)] or by the use of light petroleum ether for extraction and aluminum oxide as an adsorbent [Booth (45)]. The antioxidant may be less effective for carotene preservation than has been previously assumed (44). Difficulties in carotene estimation, particularly the quantitative extraction of carotene from chromatographic columns, have been emphasized (46) and methods described for determination of both carotene and xanthophylls in the same alfalfa extract (47). Collaborative studies on carotene and vitamin A determinations in feeds are presented (48, 49), together with suggestions for xanthophyll determinations (50). Nunez (51) has reported the separation of carotenes and various xanthophylls by paper chromatography.

Distribution.—Kon (52), in an interesting paper on carotenoids and vitamin A in marine invertebrates, includes considerable information on the amount of vitamin A in various organs of marine invertebrates and in parts of the intestine of the whale.

The amount of carotene and vitamin A in milk increases with the increase in the fat globule surface to volume ratio; this suggests that the vitamins are concentrated on the globule surface (53). Electrophoretic separation techniques have shown that carotene is associated with blood plasma proteins in approximately the following distribution: β-globulin 50 per cent, albumin 20 per cent, α_1-globulin 7 per cent, and α_2-globulin 10 per cent (54).

Bressani *et al.* (55) report that β-carotene accounts for less than $\frac{1}{2}$ the vitamin A activity of various Guatemalan corns; most of the activity not accounted for by β-carotene is attributable to cryptoxanthol.

Studies of vitamin A deficiency.—Warkany (56) reviewed the disturbance of embryonic development by maternal vitamin deficiencies, including the important studies of his group on congenital malformations in the offspring of vitamin A-deficient rats. Miller *et al* (57) suggest that hydrocephalus in vitamin A-deficient rabbits results from a primary central nervous system lesion and not from a secondary change to disproportionate growth of the skeleton and central nervous system. Incipient vitamin A deficiency in the rabbit interfered with normal implantation and fetal development although the dams appeared in good health (58).

Vitamin A deficiency in the rat has little effect on the free amino acids (59), and transaminase activity (60) of tissues increases slightly the excretion of total phenols (61) and does not impair collagen formation (62). The latter results do not support the view that scurvy accompanies avitaminosis A in the rat. The relation of vitamin A to endochondral ossification is clarified by the demonstration that administration of vitamin A to A-deficient rats lowers serum inorganic sulfate to normal levels, increases the abnormally low uptake of sulfate-S^{35} in epiphyseal cartilage as well as in sulfomucopolysaccharides of the skin, and increases the phosphate-P^{32} uptake in the diaphysis [Dziewiatkowski (63)]. A marked increase in the kidney, lung, and brain but not the liver phosphatides of vitamin A-deficient rats has been reported (64). Preliminary studies on vitamin A deficiency in cats show poor utilization of orally administered β-carotene (65).

Severe vitamin A deficiency in humans remains a problem in many areas of the world. Oomen's (66) careful studies on a group of Indonesian children with xerophthalmia and keratomalacia emphasize the need for treatment beyond the administration of vitamin A. Hills (67) reports the occurrence of vitamin A deficiency in Malayan infants fed sweetened condensed milk. Vaughan (68) believes that although xerophthalmia is manifestly rare in the United States, it may be overlooked because of lack of familiarity with early ocular signs of avitaminosis A. The serum carotene level may serve to differentiate children with and without suggested signs of vitamin A deficiency (69, 70).

Studies of vitamin A deficiency in farm animals include further reports on increased spinal fluid pressure as a criterion of vitamin A deficiency in dairy calves (71), lack of effect of level of the ration intake on the rate of depletion of plasma vitamin A and carotenoids (72), and relation of carotene or of phosphate and carotene administration to animals on rations low in both phosphate and carotene (73). Other studies include the relation of carotene feeding to reproduction in sheep (74) and range cattle (75) and reports on deficiencies in calves (76) and beef cattle (77). Important studies by Hoekstra *et al.* (78, 79) demonstrate that bovine hyperkeratosis can be produced by topical application of oil-based insecticide carriers and further emphasize the relation of the disease to deficiency of vitamin A. Observations with cattle poisoned by chlorinated naphthalenes show decreased concentrations of plasma vitamin E as well as vitamin A, [Hove (80)].

Hyperkeratosis has been produced by the feeding of rations on which toxic fungi had been allowed to grow (81).

Excessive vitamin A intake.—Gerber, Raab & Sobel (82) report a striking case of hypervitaminosis A in a young adult who consumed 500,000 units of vitamin A per day and whose condition remained undiagnosed for nine years in spite of *frequent consultation*. Acute hypervitaminosis A in the human infant appears to be without permanent harmful effects (83). Varied and frequent congenital abnormalities are found in rats when vitamin A intake during pregnancy is excessive (84). Detailed studies of the pathology of hypervitaminosis A in rats have been reported (85). An attempt has been made to relate the effects of application of large amounts of vitamin A ethers and esters on the skin of mice to the inactivation of -SH groups (86).

Utilization of carotene and vitamin A.—Interesting studies of Bieri & Pollard (87) demonstrate that carotene in aqueous dispersions when administered intravenously to rats is rapidly converted in part to vitamin A even if the bile duct is ligated or the small intestine, kidneys, or 60 to 75 per cent of the liver is removed. Thus although the intestine is the normal site of conversion of the bulk of orally administered carotene, other tissues have the ability to convert carotene to vitamin A. Related studies show that aqueous dispersions of carotene administered intravenously result in increases in plasma vitamin A values in sheep but not in Hereford calves (88). Although many investigators have assumed a direct relationship between thyroid activity and carotene conversion to vitamin A, the studies of Arnrich & Morgan (89) provide good evidence that body weight and growth rate rather than basal metabolic rate govern the utilization of vitamin A and carotene.

Extending previous studies High and co-workers show that feeding of large amounts of antioxidants decreases deposition of vitamin A when carotene but not when vitamin A is fed (90); they suggest that antioxidants may suppress oxidative steps necessary for conversion of carotene to vitamin A. Reactions involved in the biological conversion remain unknown and need further study.

Bernhard *et al.* (91) report that bilirubin and biliverdin are effective *in vitro* antioxidants for vitamin A and suggest a similar *in vivo* function may account in part for the need of bile for carotene and vitamin A utilization by rats.

Wagner (92) has reported that in man, with or without added dietary fat, up to 96 per cent of plant carotenoids are excreted in the feces. Studies with dairy cattle include a good comparison of the relative value of carotene from alfalfa and vitamin A when fed at minimum levels (93), further demonstration of the poor utilization of carotene under various conditions (94) and the effect of the form in which alfalfa is fed, on vitamin A and carotenoids in milk (95).

Calves use aqueous dispersions of vitamin A better than those in oil (96) and water-miscible forms of vitamin A when fed appear in the milk fat

as expected and not in the skim milk (97). Inclusion of sodium bentonite in feeds to improve pelleting may seriously impair the utilization of carotene and vitamin A (98). The feeding of a concentrate ration containing 30 per cent soybeans increases the carotene requirement for maintenance of adequate plasma carotene and vitamin A levels in dairy calves (99). Carotene utilization from sweet potatoes by young human adults was 54 per cent as determined by adequate balance studies (100).

Vitamin A in ethanol or in chloroform penetrates guinea pig skin rapidly by way of sebaceous glands (101); penetration is slow from oil carriers. High doses did not cause hypertrophy of the epidermis, keratinization, or hair growth.

In vitro studies of vitamin A function.—The important observations of Fell & Mellanby (102) indicative of a tissue action of vitamin A have been extended by the finding that added vitamin A prevents the keratinization that otherwise occurs in explants of rat vaginal epithelium [Kahn (103)]; the added vitamin A does not prevent but only delays the onset of cornification induced by estrogen. Weiss & James (104) feel that the action of vitamin A previously mentioned (102) may be that of inducing rather than supporting production of more normal cells.

Metabolic relationships.—Macy *et al.* (105) in a comprehensive study of the nutritional status of 1,064 women during and after pregnancy, have reported correlations of blood vitamin A and carotene concentrations with various factors.

High (106) has studied the amounts of vitamin A in tissues at various times after administration of vitamin A to A-deficient rats and during a subsequent depletion period; one finding was that plasma vitamin A values of 18 to 30 μg. per 100 ml. were attained before liver storage occurred, "indicating that physiological needs must be satisfied before vitamin A is deposited in the liver." The studies include information on the amounts of free and esterified vitamin A (107).

Kaiser *et al.* (108) present evidence that the kidney plays no significant role in vitamin A metabolism in short term experiments in the rat. Squibb, Guzman & Scrimshaw (109) found that rats subjected to 94° F. for 72 hr. showed decreases in plasma vitamin A and ascorbic acid levels.

James & ElGindi (110) present limited data showing an increase in the blood vitamin A concentration of college athletes caused by strenuous running; physical activity may be an important factor in causing irregular fluctuations of plasma vitamin A concentration.

Normal fasting human subjects showed no appreciable rise in serum vitamin A concentration 8 hr. after ingestion of 100,000 μg. of vitamin A palmitate [Mendeloff (111)]; feeding 2 hr. after vitamin A ingestion resulted in release of the vitamin to the blood. Administration of 800,000 to 1,600,000 units of penicillin has been reported to cause a decrease in human blood serum vitamin A concentration (112). A hypotensive action attributed to synthetic vitamin A has been shown to be produced by the solvent used to

carry the vitamin (113). Griesemer and associates (114) have reviewed the influence of vitamin A on the physiology of the skin; they emphasize the low value of serum vitamin A determinations in this regard and the need for development of new approaches. The chemical pathology of the carotenoids has been reviewed by Goodwin (115).

Carotene deprivation in cattle causes an increase in the amount of vitamin A alcohol in the milk and disappearance of vitamin A ester from the blood serum but does not affect the blood serum level of vitamin A alcohol; the results support the view that the metabolism of vitamin A ester is distinct from that of the alcohol and that the two substances are not in equilibrium with each other in the blood (116). Ganguly (117), now considers that vitamin A esterase activity in liver homogenates is localized in the microsomal fraction. Rousseau and associates (118) report careful studies of the relationships between plasma and liver vitamin A in dairy calves on vitamin A depletion rations.

Administration of 10,000 I.U. of vitamin A daily for 10 days to guinea pigs did not influence the cytology of the thyroid or its response to thyrotrophin (119). Herisset (120) has demonstrated what appears to be a protective antioxidant effect of carotene on various dehydrogenases in the presence of peanut oil.

Visual relationships.—These have been ably considered recently by Wald (121) and Hubbard (122) and will undoubtedly be the subject of subsequent separate reviews.

Vitamin D

Chemistry.—Inhoffen and associates have reviewed vitamin D chemistry (123) and report an excellent series of basic papers in this field. These include preparation of a new isotachysterol by treatment of vitamin D_2 with boron trifluoride-ethyl ether (124); a partial synthesis of vitamin D_2 starting with a C_{21}-aldehyde obtained by decomposition of vitamin D_2, and *p*-acetoxycyclohexanone (124); a ten-step synthesis of 2-hydroxy-4-methoxy-1-methyl-2-ethinylcyclohexene which, when reacted with a C_{19} ketone (obtained from decomposition of vitamin D_2) followed by catalytic reduction and treatment with P_2I_4 gave the methyl ether of the new isotachysterol (125); and the preparation of 8-methylhydroindan derivatives of importance in sterol syntheses (126, 127).

The synthesis of vitamin D_3-3-C^{14} has been accomplished from $C^{14}O_2$ by the necessarily laborious preparation of 7-dehydrocholestrol followed by irradiation; in particular, details are given about the irradiation conversion and isolation of the products [Havinga & Bots (128)].

Raoul *et al.* (129) report exact procedures for the conversion of vitamin D_3 into a new form which they feel may be that naturally active in organisms; vitamin D_3 ordinarily isolated from fish liver oils might arise from energetic treatment with alkali. Yoder & Thomas (130) have separated the antirachitic fraction from sulfonated cholesterol and derivatives, as the

crystalline brucine salt and present evidence that the vitamin activity observed is not the result of cleavage of the sterol molecule to vitamin D_3.

Assay.—The bioassay of vitamin D by measurement of the radioactivity in the paw of intact rats given P^{32} (131) is time-saving, objective, reliable, and has a wider assay range (2 to 20 USP units) than the line test (132). The chick bioassay may be simplified by measurement of the amount of Ca^{45} in the ash of tibiae without preliminary drying and extraction (133). The use of the ash content of the chick beak as an assay criterion is reported to be more rapid and satisfactory than the bone ash method (134). Rehling *et al.* made the pertinent observation that the oral administration of 8,000 I.U. of penicillin daily to rats approximately doubled the response in vitamin D (135); in contrast the vitamin D requirement of chicks was not changed by penicillin administration (136). Bliss and associates (137) made a careful statistical evaluation of sources in variation in the line test which demonstrated, *inter alia*, that neglect of litter-mate controls would increase by 50 per cent the number of rats necessary to obtain a given precision.

Kodicek & Ashby (138) describe briefly the separation of vitamin D and other sterols as well as tocopherols and vitamin A on filter paper impregnated with liquid paraffin and the application of procedures developed to the determination of vitamin D in materials containing more than 25 μg. vitamin D per gm. in the absence of vitamin A.

A valuable contribution by Ewing, Schlaback and associates (139, 140) describes a 2-step chromatographic process for separation of vitamin D from oils containing over 4,000 USP units per gm. and determination of vitamin D by the absorption maximum at 265 mμ; a good correspondence was obtained with the bioassay results for 110 samples. Studies are reported on the chromatographic separation of vitamin D_2 from ergosterol (141) and of vitamin D_2 from D_3 (142).

Keener has reported that the vitamin D content of forages increases with maturity and is higher for later than for early cuttings (143). Henry & Kon (144) report briefly on the vitamin D content of Indian butters.

Studies of vitamin D deficiency.—Additional reports have appeared on the occurrence of vitamin D-resistant rickets (145 to 148). Winberg and coworkers (148) emphasize that this clinical entity differs in pathogenic mechanisms from genuine rickets. Harrison (149) points out that dihydrotachysterol, which has no antirachitic activity at low dosages where vitamin D is active, may have a potency equal to vitamin D in treatment of refractory rickets and hypoparathyroidism; this suggests that the action of dihydrotachysterol in refractory rickets is by a biochemical mechanism different from that normally operative.

Winston & Pendergrass (150) report a type of osteomalacia which may be a "simple" vitamin D deficiency in adults. Poor vitamin D absorption occurs in patients with osteomalacia secondary to steatorrhea (151). Salassa *et al.* (152) showed that large doses of vitamin D favorably modified the hypophosphatemia and other changes associated with Fanconi's syndrome.

The veterinarian use of vitamin D for prevention of parturient paresis is discussed by Erdheim (153). Vitamin D may prevent the hair loss caused by heparin administration (154). Wagner (92) reports that healthy infants absorb 42 to 48 per cent of vitamin D_2 in oil compared with 29 to 39 per cent absorption by rachitic infants. The vitamin D requirements of sheep have been given particular attention by Franklin (155), Ewer (156), Green (157), and Dunlop (158).

Excessive vitamin D intake.—Cases are reported of intoxication with vitamin D in human infants (159, 160, 161) and of an idiophathic hypercalcemia in infants (162, 163). Hypersensitivity to doses of vitamin D of less than 1,000 I.U. per day may be an important etiological factor (160, 161, 162).

Frölich (164) demonstrated a growth depression in chicks fed a ration containing 1,400 I.U. of vitamin D per kg.; this was overcome by the inclusion of 30 μg. of vitamin B_{12} per kg. of ration. Neuweiler (165) found that the feeding of 1,200 I.U. of vitamin D_2 per day to guinea pigs disturbed the sexual cycle and increased infant mortality without other detrimental effects. Limited studies show little difference in the toxic response of the rat to excessive intakes of vitamin D_2 or D_3 (166). A calcinosis produced in cotton rats on certain diets was not prevented by elimination of vitamin D from the diet [Constant & Phillips (167)].

Relation to Ca and P metabolism.—Researches in this area have given further valuable information but as yet have not defined any basic causal relationships. Studies of Carlsson (168) with P^{32} in normal and vitamin D deficient rats support earlier work indicating that the absorption of P is fairly independent of the amount of Ca in the intestinal tract. That vitamin D has an effect other than on the intestinal absorption and excretion of P is clearly demonstrated by the finding that vitamin D produces the typical effects on serum inorganic phosphate concentration and on uptake of P^{32} in the skeleton of rats on a P free diet [Carlsson (169)]; evidence is also presented that the vitamin D-deficient rat cannot utilize its own bone stores for maintaining a normal serum Ca level. Jovanović *et al.* (170) conclude that vitamin D stimulates not only intestinal but also parenteral absorption of P. The results of Claassen & Wöstmann (171) show the lack of effect of calciferol when given 5 hr. before and the marked effect when given 72 hr. before P^{32} administration to rachitic rats on the uptake of P^{32} by the skeleton during the next hour. Other studies show no effect of vitamin D on the P^{32} uptake when rations with adequate Ca and P were fed (172).

Pincus and co-workers (173) report that administration of vitamin D to infants during the first week of life tends to lower serum Ca levels. Massive doses of calciferol may increase serum P and reduce serum Ca concentrations in men (174).

Hanssler (175) has reported studies on the behavior of the parathyroid glands in rickets which indicate that lower serum Ca values are associated with increased parathyroid activity. Thyroparathyroidectomy is without

effect on the production of rickets or the *in vitro* calcification of rachitic cartilage (176); the conclusion is reached that parathyroid homones are not essential for the production of the "local factor" of calcification and that diminished citrogenase activity accompanying parathyroid removal does not effect the calcification.

Relation to citrate metabolism.—Recognition of this relationship may prove to be important in the understanding of vitamin D function. Steenbock and associates (177) showed that vitamin D increased the urinary excretion of citrate by rats under a variety of experimental conditions; this effect appeared to result from an increased citrate synthesis rather than to a decrease in its destruction. Steenbock & Bellin (178) showed that physiological doses of vitamin D increased the citrate content of the blood, bone, kidney, heart, and small intestine but not of the liver of rats on normal or rachitogenic rations. Carlsson & Hollunger (179) noted an early, unexplained fall in serum citrate of rats after vitamin D administration, followed by a rise in the citrate of serum, bones, and incisors. They suggest that accelerated citrate production in the bones may account for the influence of vitamin D on the solution of bone salts. Among various aspects of vitamin D action Harrison (149) has reviewed the work of his group on increase in urinary and plasma citrate of rachitic infants following vitamin D administration. It was felt that avitaminosis D might cause a defect of specific renal tubular functions, and the group attempted to produce effects analagous to vitamin D deficiency by administration of metabolic inhibitors (149, 180); however the effects of acetazoleamide and of maleic acid on renal tubular function were not overcome by vitamin D.

Other metabolic relationships.—Havinga & Bots (128) mention briefly that after 7 months $\frac{1}{10}$ of injected vitamin D_3-3-C^{14} was still present in the chicken's pectoral muscle. Cruickshank, Kodicek & Armitage (181) studied the distribution of vitamin D in rat tissues 1 and 2 days after oral administration of 1 mg. of ergocalciferol (40,000 I.U.) Of the total vitamin D in the animal, muscle and skin contained about 20 per cent on the first day and 40 per cent on the second day. There was no evidence that organs not examined, as well as the bones, contained any appreciable amounts of vitamin D.

Carlsson considers the promotion of sulfate-S^{35} uptake by vitamin D administration to be an indirect effect of growth (182). Similarly, Dziewiatkowski found that vitamin D does not increase the rate of synthesis of chondroitin sulfate as measured with S^{35} (183); he suggests that vitamin D accelerates an impaired utilization of chondroitin sulfate. Sobel & Burgev (184) present data supporting the hypothesis that chondroitin sulfate-collagen complexes are involved in calcification. Tulpule & Patwardhan (185) suggest without convincing evidence that vitamin D deficiency results in a decreased capacity of epiphyseal cartilage to oxidize pyruvate or to metabolize glycogen.

Weits has presented further evidence that the antivitamin D factor in

forages is carotene (186). An apparent increase in the vitamin D content of silage during storage has been attributed to destruction of an antivitamin D factor [Keener (143)].

The fortification of milk with vitamin D has been reviewed by Maynard (187), and procedures have been suggested for fortification (188, 189); some irradiation procedures may be inadequate (190). Hellbrügge has recommended fortification of milk as a means of reducing the incidence of mild rickets in German infants (191). Fortification of German foods with various vitamins including A and D has been recommended (192).

Steenbock *et al.* showed that supplements with vitamin D tended to increase the extractable intestinal phytase of rats and chicks fed either rachitogenic or normal rations (193). Estrogens and corn oil are reported to give some protection against changes resulting when cockerels are fed a vitamin D deficient diet (194). A stimulation of growth by vitamin D, not the result of increased Ca retention (195), may reflect an effect of vitamin D on appetite.

Miller & Baumann (196) found considerable Δ^7-cholestenol in the skin of rats but not of other species; they suggest that it may have an accessory function as a storage reservoir for precursors of vitamin D.

Vitamin E

Chemistry.—Chemical studies on tocopherols and related compounds have been meager although there is still obscurity as to the nature and relationships of various tocopherol oxidation products. Frampton, Skinner & Bailey (197) showed that the *o*-quinone "tocored" was one of the products in addition to α-tocopherylquinone obtained by treatment of α-tocopherol in methanol with $FeCl_3$; another product is thought to be a hydroxyparaquinone. Hromatka & Kirnig (198) have reported a synthesis of mercapto-α-tocopherol by introduction of an $-SOCl_2$ or -SCN group in position 6 of desoxytocopherol, followed by reduction to the mercaptotocopherol by lithium aluminium hydride.

Fuson, Josien & Shelton (199) suggest that the apparently low frequency for the infrared cabonyl absorption of the tocopherylquinones results from an induction effect of the large number of substituents attached directly to the quinone ring.

The relation of chemical structure to biological activity in the rabbit antidystrophy and rat fetal resorption tests has been further clarified by Farber, Milman & Milhorat (200). α-Tocopheramine HCl, α-tocopheroxide, and possibly the thioacetate of mercapto-α-tocopherol showed activity near that of α-tocopherol while α-tocopherylorthoquinone was inactive in both tests. α-Tocopherylquinone, α-tocopherylhydroquinone, trimethylphythylbenzoquinone, and probably trimethyldihydrophytylbenzoquinone were active only in the antidystrophy test. The results with trimethylphytylbenzoquinone and its dihydro derivative made unlikely the possibility that antidystrophy activity depends upon conversion of test compounds to α-tocoph-

erol. The trimethyldihydrophytylbenzoquinone, thiotocopheryl acetate, and the thioacetate of mercaptotocopherol did not show antivitamin E activity.

Privett & Quackenbush (201, 202) have reported fundamental studies of the action of antioxidants in fats including tocopherol. They suggest that synergists such as phosphoric and ascorbic acids act by suppressing the antioxidant catalysis of the thermal decomposition of fat peroxides. Additional chain formation is thus prevented and the antioxidant molecules spared.

Assay.—Pollister (203) reports that formation of ethyl nitrite may interfere in the nitrosation technique for determination of γ-tocopherol; use of the water-miscible solvent dimethyoxymethane in place of ethanol obviated the difficulty. Improvement in the molecular distillation procedure for determination of vitamin E in cottonseed oil, together with vitamin E values for various cottonseed crops and products is reported by Deacon & Wamble (204). Eggett & Ward (205) give details of procedures for determination of individual tocopherols in cereal products by use of paper chromatography in an O_2-free chamber and a previously unreported tocopherol in wheat germ is indicated. Mahon & Chapman (206) suggest that adulteration of butter with vegetable oils may be detected by the resulting increase in tocopherol content. Ruccia could detect little or no vitamin E in olive oil by a rat bioassay (207).

Deficiency effects.—Mention has been made of extensive studies underway on the role of vitamin E in human nutrition [Darby & Horwitt (208)]; further reports will be awaited with interest.

Muscular dystrophy in beef cattle on winter rations low in tocopherol may be prevented or cured by vitamin E administration [Blaxter (209)]; deficient animals had less than 40 μg. compared with a normal value of 150 μg. of tocopherol per 100 ml. of blood serum. Calves fed synthetic tocopherol-deficient milks show skeletal lesions analogous to those seen in field cases of "white-muscle disease" [Safford, Swingle & Marsh (210)]. Bacigalupo & Luecke (211) noted increase of the Na and Ca in skeletal muscle and of Ca and P in heart muscle of vitamin E deficient lambs. Dystrophic symptoms in pigeons fed polished rice were prevented by vitamin E (212).

The high incidence of encephalomalacia in chicks fed "high-energy rations" became a serious problem for the broiler industry in the United States. Studies of Singsen and associates (213) give convincing evidence that the encephalomalacia of chicks which is preventable by vitamin E can be produced on practical poultry rations containing 2 per cent of a fish oil of the type commonly used as a vitamin A and D supplement. The response of chicks to vitamin E was considerably improved by concomitant administration of a mixture of vitamins C, K, and B-complex. Singsen (214) has further reported that the antioxidant N,N′-diphenyl-*p*-phenylenediamine at levels as low as 0.013 per cent of the ration prevented encephalomalacia; butylated hydroxyanisole was not effective. Ferguson, Atkinson & Couch (215) have

reported a cloudiness of the lens and other eye abnormalities, preventable by vitamin E, in embryos from hens fed a vitamin E-low diet. Slinger, Pepper & Motzok (216) confirm previous observations that vitamin E will prevent perosis in turkeys and note that addition of both vitamin E and phosphorus reduces the severity of the perosis.

Curto (217) produced testicular lesions analagous to those found in vitamin E-deficient rats by feeding a vitamin E-low diet to guinea pigs. Coujard & Daum (218) claim that the widely different histological effects of vitamin E deficiency observed in sex organs of males and females may be secondary to the same type of primary nerve lesion. Deficiencies of vitamins E and A are regarded as common causes of infertility in laboratory animals (219). Dinning *et al.* (220) observed that rats deficient in both vitamin B_6 and vitamin E exhibited a greatly increased peripheral neutrophil count prevented by supplementation with either vitamin. No anemia developed and the vitamin B_6-deficient rats exhibited a slight lymphopenia not affected by vitamin E.

Viswanatha, Gander & Liener (221) demonstrated a greater growth of vitamin E-depleted rats when fed butter fat, compared with those fed corn oil, when lactose was the dietary carbohydrate; the growth difference was not evident when vitamin E was given nor when lactose was replaced by sucrose.

Telford, Wiswell & Smith (222) showed that tocopherol deficiency decreased resistance of rabbits to decompression anoxia.

Antioxidant relationships.—Accumulated evidence adds pertinence to the statements made by Dam (223) in 1952 that, "The considerable amount of experiments available. . . shows that vitamin E exerts a very marked function in the body as an antioxidant," and, "The question remains whether vitamin E has other effects." Moore *et al.* (224) reports that uterine discoloration in rats on vitamin E-low diets is prevented by rosalinine and methyl violet but not by *p*-aminobenzoate or sulfapyridine; he is investigating the theory that some redox dyes have the antidystrophic action of vitamin E but without its power to protect the reproductive organs. Tappel (225, 226) has demonstrated that α-tocopherol and other antioxidants will prevent the *in vitro* oxidation of unsaturated fatty acids catalyzed by hematin compounds, and he suggests a similar *in vivo* biological function for vitamin E. He has further shown that the oxidation of unsaturated fatty acids in the presence of proteins gives yellow-brown polymers resembling those seen in vitamin E-deficient animals (227). Tappel & Marr (228) have discussed relationships of antioxidants, enzymes, and α-tocopherol.

Additional reports have appeared on the prevention by α-tocopherol of the deleterious effects resulting from the feeding of cod liver oil to calves (229) and pigs (230). A "yellow-fat" disease in cats produced on a commercial cat food was prevented by vitamin E (231). The potential value of fats in animal feeds and an adequate control of stability problems by use of antioxidants is discussed in a symposium. (232).

The feeding of late silage resulted in milk with the highest stability to

oxidative changes in presence of added copper although milk from cows fed early silage contained more tocopherols (233); the suggestion is made that some roughages contain substances in addition to tocopherols which protect milk from oxidative changes.

Pollister & Mead (234) found that tocopherol was effective in preventing the oxidation of linoleate induced by ionizing radiations. The possible protective effect of tocopherols against ionizing radiations in the whole animal was considered by Haley, McCulloh & McCormick (235). They found that administration of water or oil-soluble tocopherols to mice exposed to x-rays was without beneficial effects and that higher doses of vitamin E actually increased the mortality.

Hove (80) demonstrated that rats fed diets containing 0.1 per cent tri-*o*-cresyl phosphate showed an increase in conjugated dienes and an apparent decrease in arachidonic acid and tocopherols in body lipides. Supplementation with vitamin E or an increased dietary casein level prevented these changes. *In vitro* studies showed a pro-oxidant effect of tri-*o*-cresyl phosphate, carbon tetrachloride, pyridine, and pentachloronaphthalene in catalyzing the destruction of carotene in the presence of fat peroxide. The oxidative action of these substances is suggested as the cause of their toxic effects.

Absorption and distribution in the blood.—Simon, Gross & Milhorat (236) found that 73 per cent of C^{14}-D-α-tocopherol succinate given orally to a rabbit appeared in the feces in 5 days, almost all as free tocopherol. With parenteral administration there was appreciable radioactivity in the urine as well as the feces, but no free tocopherol in the urine. In a human subject on a rigidly controlled diet, 50 per cent of ingested α-tocopherol or α-tocopheryl-quinone appeared in the feces; administration with sorbitan increased the absorption about 30 per cent [Rosenkrantz, Milhorat & Farber (237)]. Increased plasma tocopherol levels in humans were noted 6 hr. after oral administration of 200 mg. or more of α-tocopherol but not after intramuscular administration of 500 mg. of tocopherol in oil or aqueous solution or as its phosphate ester (238). Orally administered α-tocopherol was more effective than α-tocopherol acetate in increasing blood tocopherol levels 6 hr. after injection (239); gastroectomized subjects showed less rise in blood levels than normals (240).

Tocopherols in human blood serum are found concentrated in the lipoprotein fractions, but, unlike carotenoids which are mainly in the β-lipoproteins, tocopherols are chiefly in the α_2-lipoprotein fraction [Lewis, Quaife & Page (241)].

Enzyme relationships.—No primary relationships between vitamin E and the activity of any enzyme system have as yet been established. Rosenkrantz (242) observed a 50 to 130 per cent increase in the *in vitro* consumption of adrenals from vitamin E-deficient rabbits before the onset of increased oxygen uptake of the muscle and of creatinuria and paralysis. Frey (243) noted a decrease in the *in vitro* oxygen uptake of rat kidney, heart, muscle, and embryonic residue following the addition of α-tocopherol phosphate,

α-tocopherol acetate, and pentamethyl-6-hydroxychroman phosphate. These effects of α-tocopheryl phosphate, as well as those reported for kidney enzymes *in vitro* (244) and others mentioned herein, cannot at present be considered to be of significance with relation to vitamin E activity; the phosphate ester may affect enzymes because of properties analagous to those of detergents which has been pointed out previously by several investigators.

Weinstock, Stoichet & Milhorat (245) noted that tissue-particulate preparations from dystrophic rabbits showed increased oxygen consumption with various substrates as compared with controls when ATP^2 was used in the medium. Replacement of the ATP by ADP^2 increased the oxygen consumption by control preparations to the level noted for those from E-deficient animals. These observations indicate that oxygen uptake was partially dissociated from phosphorylation in preparations from deficient animals; the possible significance of this will be discussed later. Previous reports have shown no difference in oxidative phosphorylation of heart mitochondria from E-deficient and normal rabbits (246).

Gray & De Luca (247) found that vitamin E deficiency in rats did not alter the glycogen formation, glucose uptake, lactic acid accumulation, or oxygen consumption of rat diaphragm. A report that vitamin E causes a lowering to normal of the levels of α-ketoglutarate, pyruvate, and citrate in the blood of cardiac patients (248) will need confirmation. The decreased activity of liver enzymes associated with a low protein diet was partly ameliorated by vitamin E (249).

Other metabolic relationships.—An interesting report by Lindahl & Kihlström (250) suggests that tocopherol may be a component of a sperm antagglutin recently described (251). Although this suggestion may be valid, the spectral and chemical properties of the substance isolated are not convincing proof of its identity with α-tocopherol.

A decreased ability of vitamin E-deficient rabbits to deposit tissue glycogen and modification of the glycogen deposition by pancreatic hyperglycemic factor have been reported (252). Administration of vitamin E does not modify the changes associated with alloxan diabetes in rabbits (253) and rats (253, 254). The disseminated muscular necrosis following experimental nephrophathy and the concomitant renal hypertension in rats on complete rations were not prevented by large doses of α-tocopherol (255).

Coxon (256) reports that vitamin E administration is without beneficial effect in the prevention of retrolental fibroplasia of premature infants; this condition appears to result primarily from a high oxygen tension in infant incubators (256, 257). MacKenzie (258) provides evidence that in premature infants a critical level of serum tocopherol exists above which complete protection is achieved against hemolysis by added H_2O_2.

Klatskin (259) has reported studies of the absorption, utilization, and storage in cirrhosis of the liver, hepatitis, and obstructive jaundice. The data show an association of low plasma tocopherol levels with liver diseases but indicate the cause to be increased breakdown of tocopherol in the intestinal

tract rather than impaired absorption. Conversely, Hilman, Nerb & Hertz (260) reported that blood levels of tocopherols were not affected by liver diseases.

Supplementing typical Danish winter rations with a gram of α-tocopheryl acetate per day did not increase milk or milk fat yield or improve the keeping quality of the butterfat from dairy cattle. (261).

Reports have appeared on the lack of effect of vitamin E on the blood clotting mechanism (262), thrombin inactivation by α-tocopheryl phosphate (263), action of α-tocopheryl phosphate on the isolated mammalian heart (264), and the relation of tocopherol to kidney clearance (265).

Essential Fatty Acids

Chemistry, assay, and distribution.—A potentially useful contribution by Howton, Davis & Nevenzel (266) is the synthesis of 1-C^{14}-linoleic acid by replacement of the original carboxyl group through bromine degradation of the silver salt reformation of the carboxyl via the Grignard reaction. The sensitive *cis-cis*-1,4-diene moiety was protected where necessary by bromination. Reinius & Turpeinen (267) obtained a small amount of C^{14}-labeled linoleic acid from yeast grown on C^{14} labeled sugars.

Thomasson (268, 269) has advanced the hypothesis with strong supporting evidence that essential fatty acid activity requires the presence of 2 double bonds at the 6:7 and 9:10 positions numbered from the terminal CH_3 group. Assay of a number of fatty acids has supported this concept, and three new compounds with essential fatty acid activity were found, namely $\Delta^{10,12}$-nonadecadienoic, $\Delta^{11,14}$-eicosadienoic, and $\Delta^{6,9,12}$-octadecatrienoic, all with double bonds at the 6,9(term) positions. Stearolic acid, $CH_3(CH_2)_7C \equiv C(CH_2)_7COOH$, which does not conform to the hypothesis and which had been reported to have activity, was found to be inactive (268).

Recent developments in oxidative deterioration of lipides have been reviewed by Lea (270). Tappel (271) has pointed out the importance of linoleate oxidation catalyzed by hemin compounds in oxidative racidity. Data of Pollister & Mead (234) on destruction of vitamins during radiation-induced autoxidation of methyl linoleate may be pertinent to radiation sterilization of foods. Several papers have appeared on the chemistry of linoleate oxidation (272 to 275).

Simmons & Quackenbush (276) achieved chromatographic separation of unsaturated fatty acids as the 2,4-dinitrobenzenesulfonyl chloride addition products; saturated fatty acids do not react with the reagent. A chromatographic method for estimation of oleic and linoleic acids is reported by Crombie, Combes & Bautman (277). Firestone (278) has reported on methods of analysis using ethylene glycol, glycerol, and alkali isomerization.

A survey of possible animal sources for highly unsaturated fatty acids by Holman & Greenberg (279) showed hog liver lipides to be the best source for isolation of arachidonic acid. Shorland (280) reports that the C_{20} unsaturated acids of animal fat are not entirely arachidonic acid. Willard,

Englert & Richards (281) give the essential fatty acid contents for a number of processed foods and hydrogenated oils. The occurrence and nutritional importance of positional and stereoisomers of the unsaturated fatty acids in hydrogenated vegetable oils has been reviewed by Melnick & Deuel (282). Estimations of unsaturated fatty acids in milk have been made by alkali isomerization (283). The diene and triene content of milk was doubled when cows were placed on summer pasture (284). Little or no essential fatty acids are reported to be present in mammary-gland fat (285).

A number of papers have appeared giving values for unsaturated fatty acids in various fats (286 to 298).

Physiology.—Deuel (299) in a comprehensive review on the nutritional significance of fats, includes a good discussion of the essential fatty acids.

Aaes-Jorgensen & Dam (300) in studies of the influence of the level and type of fat in diets of rats, produced symptoms of essential fatty acid deficiency in rats fed diets containing 7 or 28 per cent hydrogenated peanut oil; they did not find an increased requirement of linoleate with a higher level of dietary fat and noted that use of raw skim milk in place of water as a drinking fluid gave growth promotion equal to that obtained with linoleic acid. Deuel, Martin & Alfin-Slater (301) report that the rat requires up to 200 mg. of cottonseed oil per day for optimum survival of the young and for lactation; the necessary constituents of the oil appear to be essential fatty acids.

Reid has produced essential fatty acid deficiency in the guinea pig (302). Symptoms ascribable to fatty acid deficiency have been produced in calves (303) and in lambs (304) on low fat diets. Short chain fatty acids appear to be important in calf nutrition (305).

The omission of dietary fat results in gross and histological changes in the skin of dogs preventable by replacement of 29 per cent of the sucrose calories by fat [Hansen, Sinclair & Wiese (306)]; the results presented do not include prophylactic or curative experiments with essential fatty acids themselves. A report suggesting further studies gives data on the alteration of the ultraviolet absorption of epidermal material associated with fatty acid deficiency [Carruthers & Suntzeff (307)].

Important studies with C^{14}-acetate by Klenk (308) and by Mead, Steinberg & Howton (309) have confirmed the obvious hypothesis that the C_{20} and C_{22} polyene acids are derived metabolically from C_{18} precursors of linoleic or linolenic acid type by addition of C_2 units to the carboxyl end.

Fractionation and determination of serum fatty acids promises to become an increasingly important nutritional tool. On basis of measurements with 93 human infants and children on diets containing about 3 per cent linoleic acid in the fatty acids, Wiese, Gibbs & Hansen (310) have reported that serum fatty acids were 30 per cent dienoic, 1.5 per cent trienoic, and 10 per cent tetraeonic. Low serum values for linoleic acid have been reported for calves (303) and sheep (304) on low fat diets.

Data of Klein & Johnson (311) show no apparent change of di-, tri-,

tetra-, or pentaenoic acids in liver cytoplasmic particles during growth, maturation, or senescence of rats fed adequate levels of essential fatty acids; with a lower esential fatty acid intake, adequate for good growth and reproduction, an increase in linolenic and a decrease in linoleic and arachidonic acids were noted after 11 months. Additional studies (312) showed that in rats placed on a fat-free diet the concentration of unsaturated fatty acids in the cytoplasmic liver particles declined by 6 weeks to a low, nearly constant level; losses were greatest in the microsomal fraction and least in the mitochondrial fraction. These alterations preceded by about 6 weeks the onset of external symptoms, leading to the suggestion that the primary lesion may take place earlier than heretofor suspected. Johnson, Levin & Albert (313) have related changes in unsaturated fatty acid content to mitotic changes in regenerating rat liver. Liver regeneration in rats after partial hepatectomy is reported to be accelerated by essential fatty acids (314).

Alfin-Slater *et al.* (315) found that weanling rats on low fat diets showed pronounced increase in cholesterol ester concentration in the liver; they postulate that essential fatty acids are necessary for proper metabolism of cholesterol esters.

Careful studies by Klein & Johnson (316) have shown a decreased efficiency of oxidative phosphorylation by liver mitochondria from rats deficient in essential fatty acids. The possible significance of this observation is discussed in the section on vitamin K.

Deuel and associates (317) have shown a protective effect of ethyl linoleate against multiple sub-lethal doses of x-rays; the dosage for optimum protection in male rats probably exceeds 100 mg. per day. Other factors affecting the protective action are also reported (318). A diet high in unsaturated fatty acids had an aggravating action and one high in saturated fatty acids a protective action against the toxic and diabetogenic effects of alloxan (254).

Studies of Carver & Johnson (319) indicate the presence of a growth factor for chicks in the saponifiable fraction of unsaturated fats; the factor was destroyed by hydrogenation. Methyl linoleate, methyl linolenate, and methyl oleate produced inconsistent growth responses with the diet used.

The relation of fatty acids to growth of microorganisms will not be considered here; the subject has been reviewed by Nieman (320). Nutritional studies with fatty acid peroxides and oxidized fats [see for example (321, 322, 323)] will probably will become increasingly important.

Vitamin K

Chemistry and assay.—In outstanding contributions, Isler, and coworkers (324, 325) report the synthesis and action against coumarin derivatives, of 24 compounds related to vitamin K_1 in which the nature of the substituent in position 3 was varied. Activity was assessed by measurement of prothrombin time and by a rat survival test. The activity varied with type of animal as well as the coumarin derivative, especially for preparations

containing phenyl side chains. Methyl branching and double bonds in the side chain promoted activity but were not essential. Derivatives with less than 8 carbon atoms in the side chain were without activity. Vitamin K_1 showed full activity in all tests.

Valuable improvements in the synthesis of vitamin K_1 are given by Hirschmann, Miller & Wendler (326) and by Isler & Doebel (327). The latter achieved the total synthesis of vitamin K_1 through condensation of 2-methyl-1,4-naphthohydroquinone with synthetic isophytol in the presence of boron trifluoride-diethyl ether. Identity with the natural product in configuration and biological activity was adequately demonstrated.

Useful fluorescence tests for identification of naphthoquinones on paper chromatograms are described by Green (328). Identification and chromatographic separation of naphthoquinones as the 2,4-dinintrophenylhydrazine derivatives is reported by Canady & Roe (329).

Action in blood coagulation.—An important development associated with increased understanding of the blood coagulation mechanism has been the demonstration that the principal action of vitamin K and of anticoagulants is on the blood concentration of factor VII (or proconvertin) rather than on that of prothrombin (325, 330 to 336). The excellent symposium papers by Koller (333) and Jurgens (325) give details on the physiology, pharmacology, and clinical significance of blood coagulation and the relation of vitamin K thereto. A brief report, which if confirmed will be of definite interest, is that liver mitochondria incubated with factor VII and vitamin K form prothrombin with utilization of factor VII (337).

Another development of keen interest has been the well-substantiated finding that vitamin K_1 is more effective than naphthoquinone derivatives such as menadione which lacks a long side chain in position 3, in the prevention or treatment of the effects of anticoagulants in humans, rats, and chicks (324, 325, 331, 332, 333, 338, 339, 340). However, 2-methyl-1,4-naphthohydroquinone dicalcium phosphate (synkavit) was as effective as vitamin K in decreasing the prothrombin time of newborn humans when given orally to the infant [Dyggve, Dam & Søndergaard (341)] and more effective than vitamin K when given antipartum to the mother (342). The latter observation may reflect a resistance of the placenta to transfer of natural forms of vitamin K (342). Rare earths have *in vitro* anticoagulant action inhibited by vitamin K (343).

Other metabolic relationships.—Dam and co-workers have noted levels of vitamin K_1 in the blood and various organs of chicks after administration of massive doses (344). Quick, Hussey & Collentine (345) have determined the vitamin K_1 requirements of dogs and demonstrated the importance of bile for vitamin K_1 absorption; simpler substitutes for vitamin K_1 may be relatively well absorbed in the absence of bile. Hoskin, Spinks & Jacques (346) have reported the interesting finding that 2-methyl-C^{14}-1,4-naphthoquinone given in doses up to 11 mg. per kg. to rats and guinea pigs is excreted in the urine chiefly as the diglucuronide, partly as the monosulfate, and also as an unidentified derivative.

A hemorrhagic condition in chickens on practical poultry rations appears to be preventable by vitamin K (347 to 350); contributing conditions are the use of wire screen floors which prevent coprophagy, use of solvent-extracted soybean oil meal, and simultaneous use of antibiotics which reduce intestinal synthesis of vitamin K. Attention has been called to the possibility that factors other than vitamin K may be involved because of the complex pathology and aplastic anemia accompanying the syndrome (351). The decrease in prothrombin levels of human patients given antibiotics has been reported (352); this possible detrimental effect of antibiotics deserves more consideration.

The usefulness of response to vitamin K as a test for liver function has received further support (333, 353, 354). Various clinical relations of anticoagulants are considered by Koller (333) and Jurgens (325), and the bacteriostatic action of K vitamins has been reviewed by Simonnet (355). Relationships of vitamin K to endocrine glands (356), oxygen consumption of rabbit myocardium in presence of diphtheria toxin (357), and to membrane potentials of toad capillaries (358) have been reported. Menadione administration to cows appears to decrease occurrence of copper-induced off flavor (359).

Enzyme relationships.—Martius & Nitz-Litzow show that dicoumarol and related compounds can uncouple oxidation and phosphorylation (360) and decrease the oxidative phosphorylation by mitochondria from livers of vitamin K deficient chicks (361). Reports mentioned earlier have shown decreased oxidative phosphorylation in cell particulate preparations from rats with vitamin E deficiency (245) and essential fatty acid deficiency (316). Suggestions that these results are attributable to a specific function of the nutrients concerned in oxidative phosphorylation should be accepted with extreme caution. A proposal of an involvement of vitamin K in photosynthesis (362) appears even more tenuous. The ability of cell particulate fractions to carry out oxidative phosphorylation is capricious and readily inhibited by a wide variety of agents. That deviations from the normal physiological status of the animal may markedly affect the amount of oxidative phosphorylation obtained, is indicated by the observations of Potter *et al.* (363). They found that liver mitochondria from fasting rats develop ATP-splitting activity, characteristic of preparations poor in oxidative phosphorylative ability, much more readily than do mitochondria from fed rats. The decreased ability of particulate fractions to carry out oxidative phosphorylation in the *in vitro* tests used could well be a secondary effect to any of a variety of pathological conditions. Various deficiencies may interfere with oxidative phosphorylation *in vivo*, but this is not yet supported by convincing evidence.

An enzymic reduction of menadione by reduced diphospho-pyridine nucleotide, apparently distinct from quinone reductase activity, has been obtained in *Escherichia coli* and other microorganism [Wosilait & Nason (364)]. A similar activity for vitamin K_1 and other quinones has been reported in a preparation from liver mitochondria by Martius & Strufe (365, 366).

Reduction of the quinones is inhibited by dicoumarol (364, 365). The enzyme in microorganisms appears to be a flavoprotein (367, 368). The reduced quinones will in turn react, possibly nonenzymically, with cytochrome-*c* (368). Martius (366) presents evidence that the reduction occurs via cytochrome-*b* in the liver enzyme preparation and proposes that the reaction is coupled with phosphorylation; in the presence of dicoumarol or "uncoupling" agents, electron transfer is regarded as proceeding by an alternate, nonphosphorylating pathway. These interesting findings serve to re-emphasize the possible oxidation-reduction function of vitamin K and to indicate a manner in which this may be related to oxidative phosphorylation. However, the evidence to date is only suggestive and thus these interpretations must be accepted with reserve. Further research in this area is needed.

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CAROTENOIDS[1,2]

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The first article in the *Annual Review of Biochemistry* dealing exclusively with carotenoids appeared as recently as 1952 [Mackinney (1)]. It is a demonstration of the increasing interest and activity in this sphere of biochemistry that the Editorial Committee has considered a further review necessary only three years later. The extent of this activity is further emphasized by the fact that in order to keep within the allotted space and, as far as possible, to eschew cataloging, the reviewer has been forced to deal selectively with the information available. Inevitably, the topics selected for discussion reflect the reviewer's main interest.

In 1952 Mackinney could conclude with justification that the total synthesis of representative carotenoids marked the end of a chapter in organic chemistry. The immediately following section of this review will show that a new theme is now available for a further chapter in organic chemistry, the elucidation of the structures of many new and peculiar carotenoids, especially xanthophylls, which biochemists are unearthing almost daily in their, at present, frustrating search for a rationale of carotenoid distribution in Nature.

CHEMISTRY

General

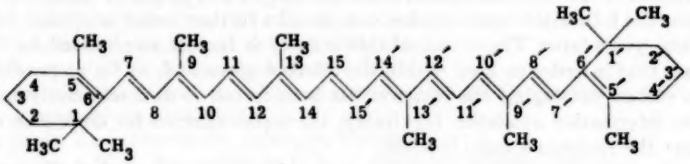
Zechmeister (2) has recently reviewed the stereoisomerization of polyenes and briefly reported new observations on the infrared absorption spectra of these isomers. A strong band at 12.84 μ is observed with 15,15′-mono*cis*-β-carotene (see Formula I for general numbering of carotenoids) but not with all *trans*-β-carotene or any other all *trans* carotenoid examined. Every *cis*-carotenoid examined except 15,15′-mono*cis*-β-carotene exhibited a band of variable intensity at 7.25 μ.

It was originally concluded that *trans*→*cis* rotation could not occur at every double bond in a carotenoid molecule, for when a carbon atom (α-carbon) adjacent to a double bond carries a methyl group, a *cis*-configuration entails considerable overlap of this methyl group and the hydrogen atom on the carbon at the other end of the double bond. Thus, rotation round carbons 11 and 12 and 7 and 8 should be hindered. Carotenoids with hindered *cis*-configuration have, however, recently been synthesized. (See Weedon (3) for further references.) Assumption of hindered configurations produces

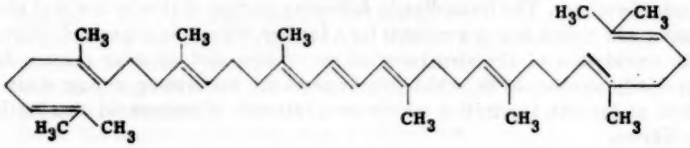
[1] The survey of the literature pertaining to this review was concluded in December, 1954.

[2] The following abbreviations are used in this review: AMP for adenylic acid; DPA for diphenylamine.

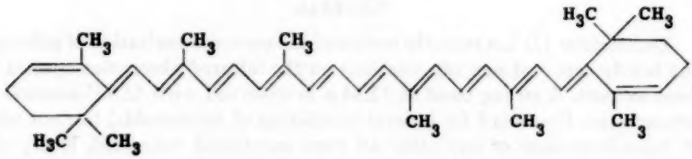
a much greater change in the absorption spectrum of the parent all *trans* compound than does a change to a "permitted" *cis*-configuration; e.g., the change from all *trans*-β-carotene to 11,12-mono*cis*-β-carotene not only shifts the main absorption maximum from 470 to 433 mμ (light petroleum), but also causes a marked loss in definition of the spectrum. The introduction of two hindered *cis* bonds (11, 12 and 11′, 12′) results in the complete suppression of the characteristic fine structure in the spectrum of all *trans*-β-carotene [Garbers & Karrer (4); Eugster *et al.* (5, 6)].



Formula I. General numbering of carotenoids.



Formula II. Phytoene.



Formula III. Dehydro-β-carotene.

Zechmeister (2) does not consider these hindered forms necessarily thermolabile in spite of their high energy content; they are, however, exceptionally sensitive to I_2, quickly disappearing from I_2-treated solutions. The poly*cis*-lycopenes observed naturally in *Pyracantha* berries [Zechmeister & Pinckard (7)] may be "hindered" isomers because they do not occur in heat isomerized lycopene solutions [Zechmeister & Petracek (8)] and because the absorption spectra are very similar to those of some synthetic "hindered" *cis*-lycopenes [Garbers & Karrer (9)].

Porter (10) has deduced a formula $(\lambda/100)^2 = 0.3498n' + 2.9754n - 0.7955n^2$, relating the longest wave length (λ in Å) absorption maximum with the total number of conjugated double bonds (n) and the number of

—C(C)=C groups (n') in the system. Although only limited data were available for analysis, relationships also appear to exist between n and m.p. and n and R_F values. Dale (171) has recently discussed theoretical aspects of the minor absorption bands in the spectra of polyenes.

Hydrocarbon Polyenes

Colourless polyenes.—As more materials are being examined in greater detail for carotenoids, many new polyenes, especially hydrocarbons, are being described. Because of their occurrence in trace amounts, most of these new polyenes have not been crystallized, but so much information is now available concerning the absorption spectra and relative positions on chromatographic columns of most carotenoids, that it is reasonable at this stage to accept the individuality of these new polyenes in spite of the absence of an important desideratum of classical organic chemistry.

Isolation and purification of phytoene on a comparatively large scale [Rabourn *et al.* (11)] has allowed degradative studies to be carried out which reveal 7,8,11,12,12′,11′,8′,7′-octahydrolycopene (see Formula II) as its structure [Rabourn & Quackenbush (12)]. Evidence has also been obtained from the same laboratory that phytofluene is not a dehydrophytoene but an isomer in which the two double bonds at 9,10 and 9′,10′ have moved into conjugation with the central unsaturated system, i.e., phytofluene is 7,8,9,10,10′,9′,8′,7′-octahydrolycopene [Rabourn & Quackenbush (private communication)]. It should be remembered, however, that Koe & Zechmeister (21) apparently obtained phytofluene from phytoene by dehydrogenation with *N*-bromosuccinimide. Phytoene has been detected for the first time in chlorophyllous tissues, i.e., young alfalfa [Rabourn & Quackenbush (13)] and *Hevea brasiliensis* [Eny (14)]. It is also present in the latex of *H. brasiliensis* and in a number of fruit but is absent from green tomatoes, lemon grass, lettuce, dried alfalfa, lemons, and avocados. Phytofluene has also been recently detected in the green tissues and latex of *H. brasiliensis* (14) and in the leaves of a large number of plant species representing 18 families. The amounts present in the latter are extremely small, varying between 0.012 and 0.36 mg./kg. (fresh weight) [Zechmeister & Karmakar (15)].

The naturally occurring phytofluene in tomatoes is a *cis*-isomer [Petracek & Zechmeister (16); Koe & Zechmeister (17)]; the *cis* peak, which can only be observed with highly purified samples, occurs at about 251 mμ. The difference between this and the longest wave length band is 115 mμ, which agrees with that found for the 1,4-diphenylbutadienes [Pinckard *et al.* (18)] but is less than that normally associated with carotenoids (2). Phytofluene is relatively thermostable, but when exposed to light 90 per cent is converted into *trans*-phytofluene, which is adsorbed more strongly than phytofluene and has an absorption spectrum in almost the same position but with slightly greater definition. Its $E^{1\%}_{1cm.}$ value is 1600, while that for phytofluene is 1350. The $E^{1\%}_{1cm.}$ value of 1520 recorded by Wallace & Porter (19) for phytofluene, was probably the result of the production of some *trans*-phytofluene during

their isolation procedures. Trombly & Porter (20) have observed in various tomato crosses "colourless polyene A" which is very similar to *trans*-phytofluene but considered different from it because it is adsorbed above and not below α-carotene.

By treatment with *N*-bromosuccinimide, both phytoene and phytofluene can be converted into a mixture of coloured polyenes having absorption spectra corresponding to carotenoid systems containing 7,9,11, and, probably, 13 conjugated double bonds. Some have *trans* and some *cis*-configurations. When phytoene is the starting material phytofluene is also formed [Koe & Zechmeister (21)]. Squalene, which can legitimately be considered a partly saturated polyene also yields coloured products with *N*-bromosuccinimide [Dale (22)]. Lijinsky & Zechmeister (173) have recently reported on the polyenes produced by the partial catalytic hydrogenation of lycopene.

Nakayama *et al.* (23) obtained two colourless compounds, the second a polyene, from the yeasts *Rhodotorula peneaus* and *Cryptococcus laurentii*, neither of which corresponded with phytofluene. One, weakly adsorbed, fluoresced blue in ultraviolet light and had an absorption maximum at 340 mμ with inflexions at 325 and 358 mμ, while the other, more strongly adsorbed, fluoresced green (λ_{max} 365, 385, and 405 mμ). Links *et al.* (24) have also recently briefly reported the presence of colourless polyenes different from phytoene and phytofluene in the alga *Polytoma uvella*. A new slightly straw coloured polyene (λ_{max} 350, 369, and 390 mμ in light petroleum), adsorbed between phytofluene and ζ-carotene, has been detected in Valencia oranges [Nataradan & Mackinney (25)].

β-Carotene.—The action of *N*-bromosuccinimide on β-carotene produced (*a*) dehydro-β-carotene (isocarotene) (m.p. 192–193°C.) (see Formula III), (*b*) a new pigment, bisdehydro-β-carotene (m.p. 204°C., λ_{max}, hexane 457, 490, 521 mμ) together with two isomers, and (*c*) probably anhydroeschscholtzxanthin (2,3,2′,3′-dehydro-*iso*-carotene) [Zechmeister & Wallcave (26)]. Zechmeister & Wallcave (27) also found that the conventional (I_2) method for producing dehydro-β-carotene yields a mixture of at least nine *cis* isomers. Neodehydro-β-carotene J which is obtained only on melting the crystals of the all *trans* compound is somewhat unique. It exhibits a single absorption band (λ_{max}, hexane, 449 mμ) and on treatment with I_2, its yellow solution immediately turns intense orange-red, a reaction reminiscent of that of the prolycopenes [Zechmeister *et al.* (28)]. BF_3 reacts rapidly with dehydro-β-carotene to form a dark blue complex which is decomposed by water to yield 4-hydroxy-β-carotene (*iso*cryptoxanthin) (m.p. 162–164°C., acetate 108°C.; absorption spectrum almost identical with that of cryptoxanthin). If the BF_3-dehydro-β-carotene complex is disrupted with CH_3OH instead of H_2O, *iso*cryptoxanthin methyl ether (m.p. 133–135°C.) results together with traces of a structurally uncertain dimethoxy-β-carotene (m.p. 152°) [Wallcave & Zechmeister (29)]. *Iso*cryptoxanthin and cryptoxanthin can be separated chromatographically only with considerable difficulty; they are easily differentiated however, by the fact that *iso*crypto-

xanthin is converted to dehydro-β-carotene when treated with chloroformic HCl. The $CHCl_3$/HCl reaction occurs when a carotenoid contains the grouping —CH(OH)·CH=CH—. These experiments incidentally, confirm the structure of cryptoxanthin as 3-hydroxy-β-carotene [Karrer (30)].

Other carotenes.—New pigments found in tomato crosses by Trombly & Porter (20), included pigment A (λ_{max} 415, 439.5, 470 mμ in hexane), an all *trans*-carotene adsorbed between phytofluene and phytoene; pigment C (λ_{max} 435, 460.5, 491 mμ in hexane) and its *cis*-isomer pigment B (λ_{max} 436.5, 457.5, 486.5 mμ in hexane). Neurosporene (tetrahydrolycopene) was also observed in tomatoes for the first time, and it was concluded that the pigments previously designated "Unidentified I" [Porter & Zscheile (31)] and poly*cis*-ψ-carotene [Mackinney & Jenkins (32)] are identical with protetrahydrolycopene.

The shoulder at 452 mμ observed in the absorption spectrum of "ζ-carotene" from *Phycomyces blakesleeanus* [Goodwin (33)] but which did not occur in ζ-carotene from other sources [see e.g. (31)], is attributable to a pigment (abs. max., hexane, 421 and 452 mμ) slightly less strongly adsorbed than ζ-carotene [Goodwin & Osman (34)]. K-carotene [White *et al.* (35)] and "carotene I" [Baumgarten *et al.* (36)] both reported in corn, are probably also a mixture of ζ-carotene and this second pigment, which also occurs in diphenylamine (DPA) cultures of *Mycobacterium phlei* [Turian & Haxo (37)], *Allomyces javanicus* [Turian & Haxo (38)], and certain mutant strains of *Neurospora crassa* [Haxo (39)]. It appears to be very similar to flavacin first obtained from the blue green alga *Aphanizomenon flosaquae* [Tischer (40)].

A pigment, η-carotene, with an absorption spectrum similar to that of ζ-carotene, but with weaker adsorptive properties occurs in the berries of *Lonicera japonica* [Goodwin (41)] and probably in the roots of *Brassica rutabaga* [Joyce (42)]; the latter also contain a number of new poly*cis*-lycopenes.

The photosynthetic bacterium *Rhodospirillum rubrum* when cultured in the presence of DPA,[3] synthesizes very little of its normal carotenoid spirilloxanthin, but small amounts of a complex mixture of polyenes [Goodwin & Osman (43)], including phytofluene, ζ-carotene, neurosporene, and their monohydroxy derivatives (80).

Xanthophylls

Strain (44) has demonstrated that the second most abundant xanthophyll in green leaves is violaxanthin (5,6,-5′,6′-diepoxyzeaxanthin) and not lutein-5,6-epoxide as previously stated [Karrer *et al.* (45)] and that the violaxanthins from leaves and pansy petals are identical. Treatment with ethanol/acetic acid (30 min.) converts violaxanthin into two noninterconvertible flavoxanthin-like compounds, both of which give stable blue colours when dissolved in ethyl ether and treated with conc. HCl. Prolonged ethanol/acetic acid treatment converts violaxanthin and the flavoxanthin pigments into auroxanthin but not into mutatochrome and zeaxanthin as reported by Karrer & Jucker (46). At present it does not appear that either

TABLE I

POLYENE DISTRIBUTION IN VARIOUS ORGANISMS*

	Pigments Present (See Key p. 504)	Reference
A. PHANEROGAMS		
FRUIT		
Capsicum annuum	4, 14, 15, 17 33	60
Citrus paradisi†	2, 3, 4, 7, 9	61
Elaeginus spp.	9	174
Encephalartos longifolium	3, 4, 7, 9, 15, 33	62
E. villosus	3, 4, 7, 9, 15	62
Lonicera japonica†‡	2, 4, 5, 7, 9, 17, 33	41
Rosa polyantha	3, 9	63
Shepherdia canadensis	2, 3, 4, 5, 9, 16, 19, 24	172
Tecoma capensis	2, 4, 7, 9, 33	64
T. stans	4, 33	64
PETALS		
Calendula officinalis§‖	2, 4, 5, 7, 9, 16, 19 20, 25, 33	65
Viola tricolor§#	3, 4, 9	66
POLLEN		
*Asphodelus albus***	3, 19, 22, 34	67
Cyclamen persicum††	4, 8, 9	68
Leucojum vernum‡‡	3, 4	69
B. CRYPTOGAMS		
ALGAE		
Chlorella vulgaris‡	4, 22, 26	70
Euglena gracilis v. *bacillaris*	4, 22, 26	71
E. gracilis v. *fuscopunctata*	4, 22, 26	71
Haematococcus pluvialis§§	4, 11, 22 (?26)	72
Heterococcus fuorensis	4, 22, 26	73
Ophiocystis major	4, 22, 26	73
Polytoma uvella	4	24
Ulva lactuca (gametes)	3, 4, 7	74
Tribonema aequale	4, 22, 26	74
FUNGI		
Allomyces javanicus‡	4, 7, 9	38
Blastocladiella emersonii (mutant)	7	75
Cryptococcus laurentii‡	4, 7, 10	23
Cryptococcus luteolus‡	4, 7, 10	23
Dacromyces stillatus	2, 3, 4, 5, 17, 31, 33	76
Gloeosporium musarum	13	73
Monolia sitophila	4	77
Neurospora crassa (mutants)	2, 3, 4, 5, 6, 8 9, 21, 24, 27, 29	39
Rhodotorula glutinis‡	4, 9, 30, 31	23

TABLE I (*continued*)

	Pigments Present (See Key p. 504)	Reference
Rhodotorula rubra‡	4, 9, 30, 31	23
BACTERIA		
Corynebact. michiganese (parent)	9, 17	78
C. michiganese (mutants)	4, 9, 13, 17, 28	78, 79
Chlorobium‖‖ spp.	7	80
Chromatium spp.	9, 23, 24, 29	80
Mycobact. phlei	3, 4, 7, 9	56, 81
Staphylococcus aureus	9, 22	82
C. PROTOZOA		
Prorocentrium micans	4, 35	83
D. ANIMALS		
MARINE INVERTEBRATES		
Asterias rubens	4, 11	84
Asterina pectenifera	11	175
Echinaster sepositus	4, 17	85
Reniera japonica	4, 10	176
AMPHIBIA		
Triton nodifer	11	86
INSECTS		
Amphipyra sanguini punctata♯♯	4, 22	87
Cacoecia australana♯♯	4, 22	87
Cassida murraea•••	4	88
Nezara viridula♯♯	4	87
Pieris rapae♯♯	4, 11	87
Plusia gamma♯♯	11	89
BIRDS (feathers)		
Laniarius atrococcineus	11	90
L. babarus	22	91
Megaloprepia magnifica	39	92
Oriolus auratus	11	91

* Only those not fully discussed in text included here.
† No xanthophylls present.
‡ See also text.
§ Only deeply coloured varieties contain lycopene.
‖ Some poly*cis*-lycopenes also present.
♯ Xanthopyllls also present (see 59).
** Also two unidentified red carotenoids.
†† Also probably dehydro-β-carotene.
‡‡ The carotenes are in the outer wall and xanthophylls in the inner.
§§ Astaxanthin present only in encysted cells.
‖‖ Plus traces of unidentified polyenes.
♯♯ Haemolymph.
••• Integument.

KEY TO PIGMENTS

TABLE I (*continued*)

1. Phytoene	13. Canthaxanthin	25. Mutatochrome
2. Phytofluene	14. Capsanthin	26. Neoxanthin
3. α-Carotene	15. Capsorubin	27. Rhodopurpurin
4. β-Carotene	16. Chrysanthemaxanthin	28. Rhodovibrin
5. ζ-Carotene	17. Cryptoxanthin	29. Spirilloxanthin
6. δ-Carotene	18. Echinenone	30. Torularhodin
7. γ-Carotene	19. Flavochrome	31. Torulin
8. Neurosporene	20. Flavoxanthin	32. Violaxanthin
9. Lycopene	21. Hydroxy-γ-carotene	33. Zeaxanthin
10. Leprotene	22. Lutein	34. Peridinin
11. Astaxanthin	23. Lycophyll	35. Lutein epoxide
12. Auroxanthin	24. Lycoxanthin	36. Rhodoxanthin

of the flavoxanthin-like pigments is flavoxanthin, which is 5,8-epoxylutein, because, accepting the structure of violaxanthin, they must be derivatives of zeaxanthin. Curl & Bailey (47) have, therefore, suggested that these two pigments, which are found in orange juice, should be named luteoxanthins *a* and *b*. Unfortunately, this perhaps suggests a relationship to lutein.

Taraxanthin treated with ethanol/acetic acid yields two chrysanthemaxanthin-like pigments (flavoxanthin and chrysanthemaxanthin are isomers apparently differing only in the spatial disposition of the two oxygen atoms at positions 3 and 5 and in the fact that the latter gives only a fleeting blue colour with ether-HCl) which on further ethanol-acetic acid treatment gave a pigment with λ_{max} (CS_2) at 420 and 450 mμ. Violeoxanthin and tareoxanthin behaved in the same way as their parent pigments, the resulting compounds having slightly lower absorption maxima than those derived from the parents.

Bickoff *et al.* (48) found that five pigments constituted 99 per cent of the xanthophyll fraction of alfalfa, viz.: cryptoxanthin (4 per cent), lutein (40 per cent), violaxanthin (34 per cent), zeaxanthin (2 per cent), and neoxanthin (19 per cent). Some of the seven additional pigments observed in traces may be artifacts, for the lability of plant xanthophylls is considerable, e.g., after standing for 48 hr. at room temperature in the dark, a solution of neoxanthin can be resolved into seven components. The xanthophyll fraction of dehydrated alfalfa could be resolved into more than 40 fractions, although the five main pigments still represented 87 per cent of the total pigments. The same major pigments occur in corn seedlings [Moster *et al.* (49)]. An interesting new xanthophyll which occurs in corn seedlings (49), alfalfa (48), and orange juice (47) appears to be hydroxy-α-carotene.

Curl & Bailey (47) combining the counter-current technique [Curl (50)] with adsorption chromatography have gone far in separating the complex mixture of carotenoid epoxides in fresh orange juice. Antheraxanthin (zeaxanthin-5,6-epoxide) and a *cis*-isomer, a group of mutatoxanthin isomers, violaxanthin and *cis*-violaxanthin (possibly identical with violeoxanthin),

auroxanthin, the luteoxanthins and *cis*-luteoxanthin are present, together with, probably, trollixanthin and trollichrome (5,6- and 5,8-epoxides of a dihydroxy-α-carotene). Among the new polyenes described were (*a*) valenciaxanthin [λ_{max} (benzene), 358, 377, and 399 mμ], first detected by Natarajan & Mackinney (25) but not named; (*b*) valenciachrome [λ_{max} (benzene) 337, 354, and 375 mμ], a furanoid epoxide with green fluorescence, reminiscent, apart from its high adsorptive power, of phytofluene; and (*c*) sinensiaxanthin [λ_{max} (benzene) 360, 380, 404 mμ] which is probably not an isomer of auroxanthin but a pigment with less than 40 carbon atoms. Acid treatment converts valenciaxanthin into valenciachrome.

De Nicola & Goodwin (51), extending the observations of de Nicola & Monroy-Oddo (52), found, apart from echinenone (4-keto-β-carotene) and pentaxanthin, two unique xanthophylls in the eggs and ovaries, but not the testes, of *Paracentrotus lividus*. These pigments, named paracentrotin A and B, have slightly unsymmetrical absorption spectra [λ_{max} (light petroleum) 488 to 490 and 480 mμ respectively]. On addition of KOH their spectra shift to shorter wavelengths and exhibit the typically triple banded carotenoid spectrum [λ_{max} (light petroleum) 435, 460, and 486 mμ and 430, 455, and 480 mμ respectively]; their adsorptive properties remain unchanged and the spectral changes are not reversed on neutralization. The structures of these pigments are at the moment difficult to envisage. It is important to re-examine the gonads of other species of sea urchins for these alkali-labile pigments, because earlier work almost invariably involved preliminary alkaline saponification of the tissues.

Fox (53) recently described briefly an esterified acidogenic carotenoid present in a bathypelagic nemertean worm *Nectomertes mirabilis*. It appears to be slightly different from astaxanthin (3,3′-dihydroxy-4,4′-diketo-β-carotene). The red skin, lips, and tongue of the opah (*Lampris regius*) contain an esterified carotenoid very similar in adsorptive and spectral properties to astaxanthin but quite different in exhibiting no acidic properties [Fox & Goodwin (unpublished observations)]. De Nicola (54) confirmed the presence of astaxanthin in *Ophidiaster ophidianus* but found in addition two new pigments which may be intermediates on a biosynthetic route from β-carotene to astaxanthin. Traces of α-carotene, cryptoxanthin, and, possibly, γ-carotene are also present. The colourless fluorescent extracts from the marine worm *Thoracophelia mucronata* were complex mixtures, yielding no known polyenes [Fox *et al.* (55)].

Two marine coryneform bacteria isolated from the skin mucus of the Arctic cod synthesize three pigments with identical absorption spectra [λ_{max} (light petroleum), 412, 437, 466 mμ], neoxanthin, sarcinaxanthin, and a very strongly adsorbing pigment which has been named corynexanthin [Hodgkiss *et al.* (56)]. Many marine bacteria are characterized by their synthesis of highly hydroxylated carotenoids of unknown structure [Courington & Goodwin (unpublished observations)].

Links *et al.* (24) have reported a carotenoid very similar to canthaxan-

thin, but differing from it in being weakly acidic, in the alga *Polytoma uvella*. Goodwin & Jamikorn (57) consider that Turian's (58) chrysophlein obtained from *Mycobacterium phlei* is a mixture of two polar carotenoids which are probably not acidic.

Studies have continued on the general distribution of carotenoids, especially minor components, in Nature, and the main observations not discussed above, are summarized in Table I. In spite of this and much previous work [see Goodwin (59) for a review] it is still difficult to generalize sensibly on the subject of carotenoid distribution.

BIOSYNTHESIS OF CAROTENOIDS

Higher Plants

General factors.—Confirmation of high carotene levels in young plants followed by a decline during maturation was obtained with orchard grass [Clarke (93)] and alfalfa [Staker & Crandall (94)]. Good correlation exists between nitrogen and carotene content in these and other forages, but not in legumes [Bressani & Johnson (95)]. When legumes are grown associated with grasses their carotene content is unaltered, but that of orchard grass increases considerably when grown associated with Ladino clover (95). Bressani & Johnson (95) also confirmed that environmental conditions affect carotene synthesis but, using brome grass, could not demonstrate a diurnal variation in carotene levels [see (59) for earlier work suggesting the existence of a diurnal variation].

Genetic studies. (General)—Clarke (93) found in the case of orchard grass, significant differences in carotene content between the parent strain and the fifth generation of inbred clones. No such differences were observed in alfalfa (94), corn [Moster & Quackenbush (96)] or celery [Wilcox *et al.* (97)].

An ultrasonic mutant of the sunflower (*Helianthus annuus*), obtained by Wallace & Schwarting (98), is devoid of carotenoids in leaf, petal, and pollen. It grafts freely and sets seed to its own pollen. It can, however, produce some chlorophyll under low light intensity.

In etiolated wheat seedlings, the carotenoid concentration increases for the first eight days, remains constant until the twelfth day and then declines rapidly. Addition of glucose causes accumulation to continue after the eighth day, but urea inhibits synthesis [Beekmann (99)].

Minerals.—Further confirmation has been obtained for the view that the N content of the soil is an important factor controlling carotene levels in plants [Pfützer *et al.* (100); Scharrer & Bürke (101)]. P and K deficiencies reduce carotene levels, but excess of either has no stimulatory effect above normal (101).

Temperature, light, and oxygen.—Corn seedlings form more zeaxanthin and less β-carotene at low than at high temperatures (96); similarly high light intensity increases zeaxanthin and reduces β-carotene production; quantitative experiments suggest that β-carotene is converted into zeaxanthin. Anal-

ogous changes occur with lutein and violaxanthin, but the postulated interconversion appears doubtful in this case because violaxanthin is a derivative of zeaxanthin and not of lutein.

It is very interesting that *Reseda odorata* leaves on strong illumination at 0°C. lose almost all their lutein whilst gaining an equal amount of rhodoxanthin [Sörensen (177)].

Carotenoid synthesis in bean and corn seedlings is stimulated by light of wavelength 725 mμ up to an intensity of 450 μ watts per sq. cm.; above this intensity synthesis drops [Withrow *et al.* (102)]. The reduced synthesis at high light intensity is 50 times greater than the simultaneous stimulation of chlorophyll synthesis, suggesting no direct biogenetical relationship between these two pigments. In etiolated wheat seedlings a concentration of 4.2 per cent O_2 in the atmosphere reduces pigment synthesis which is normal at a level of 9.5 per cent O_2 (99).

Tracer studies.—Roux & Husson (103) claimed, using C^{14}, that neither of the carboxyl carbons of glutamate was incorporated into maize plants either in the light or dark. Isolated tobacco and tomato leaves, however, incorporate $C^{14}O_2$ into β-carotene and the indications are that there is a fairly rapid turnover of the pigment [Glover & Redfearn (104)]. Under the same conditions, the methyl carbon of acetate is incorporated to a much less extent than is CO_2 [Glover & Redfearn (unpublished observations)]. It is appropriate here to emphasize that in tracer studies with β-carotene, the pigment must be obtained crystalline; spectroscopically and chromatographically "pure" β-carotene is often contaminated with traces of highly active colorless material, as yet unidentified, which cannot be removed chromatographically.

Isolated tissues.—Excised tomato leaves kept in darkness for 24 hr. in the absence of CO_2, lose considerable amounts of β-carotene whilst β-carotene epoxides accumulate. On admitting CO_2 and light, the epoxide level drops to normal almost immediately, but there is a 24 hr. lag before the β-carotene level begins to increase, attaining its original level after 96 hr. These observations suggest that epoxide formation is the first step in the oxidative destruction of β-carotene but not a step in its synthesis (104).

Inhibitors.—Streptomycin, guanidine hydrochloride, chloromycetin, *iso*nicotinic acid hydrazide, maleic hydrazide, and tetronic acid inhibit carotenogenesis in pea seedlings while penicillin is inactive. Tetronic acid is very potent, being 60 times more effective than streptomycin, and *iso*nicotinic acid hydrazide inhibits only carotenoid synthesis; all the others inhibit chlorophyll production equally [Schopfer *et al.* (105, 106)]. In etiolated wheat seedlings, low concentrations of 2,4-dinitrophenol and chloroform stimulate while high concentrations inhibit carotenoid production. When chloroform-inhibited leaves are placed in pure air the carotenoid levels return to normal within 24 hr. Malonate and trypan blue stimulate while fluoracetate inhibits synthesis; citrate but not acetate overcomes the fluoracetate inhibition (99).

Fruit (*genetic studies*).—The basic principles of studying pigments genetically have been discussed by Mackinney (1). Here it is sufficient to state that in tomatoes (the fruit almost universally used) three major genes, *R*, *T*, and *B*, have been described which control carotenoid synthesis. *R* and *T* are completely dominant [Jenkins & Mackinney (107); Tomes *et al.* (108)]. An anomaly in the F_1 progeny of normal red × high β-carotene (*B*) tomatoes [Tomes *et al.* (109)] is attributable to the presence of a modifier contributed by the red parent (108).

The problem of carotenoid biosynthesis is complex and until more is known of the fundamental reactions concerned, it is possible to define the function of these genes only in most general terms. The most plausible role of *R* is in controlling quantitatively, far back in the synthetic pathway, the production of an unknown common polyene precursor. Even on this wide basis anomalies arise, e.g., the double recessive *rrtt* has three to four times as much carotene as does *rrTT*. *R* now appears to have no specificity for lycopene synthesis as previously suggested.

T controls the chanelling of precursors into either the lycopene-β-carotene series or the ζ-carotene-prolycopene series, while *B* controls the relative amounts of β-carotene and lycopene produced. Recently Mackinney *et al.* (110) have studied a mutant of the wild tomato *Lycopersicon pimpinellifolium* discovered in the Galapagos Islands, the fruit of which produces β-carotene almost exclusively, while the mainland strain produces lycopene. Examination of the F_1 hybrid with *L. esculentum* indicates the presence in the Galapagos strain of a gene very similar to, if not identical with *B*, which is tightly linked with the self-pruning (*sp*) locus.

The Porter & Lincoln (111) scheme of carotenogenesis in tomatoes is, in the light of present work, at least an oversimplification. The present form of the main scheme [Porter (personal communication)] indicates the synthesis of lycopene by the stepwise dehydrogenation (four H atoms at a time) of a saturated precursor: tetrahydrophytoene→phytoene→phytofluene→ζ-carotene→protetrahydrolycopene→prolycopene, prolycopene then being isomerized to lycopene. Recent work suggests that phytofluene is not tetradehydrophytoene, but an isomer of phytoene [Rabourn & Quackenbush (personal communication)]; simple dehydrogenation cannot, therefore, account for the transformation of tetrahydrophytoene to prolycopene, if it occurs. Furthermore the complete process envisaged as being controlled by gene *R*, itself controlling an α-β-dehydrogenase appears unacceptable. As for the conversion of prolycopene into lycopene Jenkins & Mackinney's (112) work has led them to conclude that "there seems to be no reason to single out prolycopene as the specific precursor of lycopene."

There appears little doubt that the synthesis of β-carotene in normal red tomatoes is via a route different from that producing lycopene, for in tomatoes ripened above 30°C. lycopene (and phytofluene etc.) synthesis is inhibited, while that of β-carotene is little changed [Sayne *et al.* (113); Denisen (114); Goodwin & Jamikorn (115); McCollum (178)]; it is probable that the

β-carotene is synthesized by the same route as in green tissues. It is tempting to agree with the suggestion (111) that in the high β-carotene/low lycopene tomatoes, β-carotene is formed from lycopene, gene *B* controlling a ring-closing enzyme. Experiments involving ripening high-β-carotene tomatoes at temperatures above 30°C. might be expected eventually to settle this problem. At present, only two contradictory reports are available. Wallace [quoted by (108) found β-carotene synthesis reduced at 33°C. while Manunta (116) reported no inhibition of β-carotene but marked inhibition of lycopene at 36°C.

Roots.—The carotene content of boron-deficient carrots is significantly lower than that of normal carrots. The carotene content (on fresh weight basis) increases with increasing amounts of B added to the sand culture; on a dry weight basis the concentration does not increase after the B deficiency symptoms disappear. The low carotene levels in B deficiency is probably attributable to a disruption of carbohydrate supply to the roots rather than to a direct effect on carotenogenesis [Kelly *et al.* (117)]. Carrot roots grown in tissue culture in light and darkness do not synthesize β-carotene or colourless polyenes [Goodwin (unpublished observations)].

Microorganisms

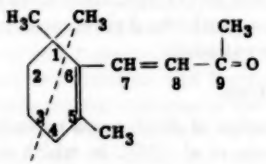
Temperature.—Low temperature cultivation of *Rhodotorula rubra* results in yellow mycelia [Skoda (118); Nakayama *et al.* (23)], in which α- and β-carotene comprises over 90 per cent of the total pigment, whilst at 25°C. they represent only 45 per cent of the total, the remainder being torulin and torularhodin. The same but less well-marked trend occurs in *R. rubra*, whilst *R. peneaus*, and probably *R. aurea*, synthesize less of all components at low temperatures (23). No qualitative differences are observed with *Phycomyces blakesleeanus*, β-carotene being the main pigment produced at all temperatures. The amount produced is, however, less at 3 to 5°C. and at 30°C. than at 20°C. [Friend & Goodwin (119)].

Turian (81) found that *M. phlei* produced predominantly carotenes at 30°C. and predominantly xanthophylls at 37°C. Certain marine bacteria produce considerable amounts of carotenoids at 20°C. (56), but very little at 30° to 37°C. [Goodwin (unpublished observations)]; transference of cultures grown at 37° to 20°C. does not result in pigment synthesis.

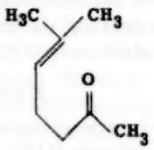
Oxygen and light.—Conidia-free *N. crassa* pads were obtained in the dark in submerged culture by adding Tween 80 to the usual medium; these cultures were virtually colorless but contained some phytoene and phytofluene. Exposure of such mycelia to light and oxygen for 1 min. was sufficient to allow full pigment synthesis in the dark and in the presence of O_2. Light without oxygen was ineffective as a stimulator, and oxygen was also a prerequisite for dark synthesis. After light stimulation, the phytoene level first drops and then increases; phytofluene remains unchanged; lycopene, ζ-carotene, neurosporene, and β-carotene reach a steady value after 48 hr., while γ-carotene, the acidic pigment, and phytoene appear still to be in-

creasing after 24 hr. [Zalokar (120)]. Light stimulates, but is not essential, for β-carotene synthesis in *P. blakesleeanus* [Garton *et al.* (121); Chichester *et al.* (66)]. In *Rhodotorula gracilis* [Praus (122)] light also stimulates pigment production, but in this case the β-carotene : torulin ratio is greater in the light than in the dark, while in *P. blakesleeanus* no qualitative changes occur in the pigments present. Dark and light cultures of *Chlorella vulgaris* produce the same carotenoids [Goodwin (70); Claes (123)], but the concentration in old cultures is greater in the dark than in the light (70).

Growth-factors.—Thiamine has a profound qualitative effect on carotenogenesis in *Corynebacterium poinsettiae;* with a concentration of 0.1 μg./100 ml. the cells are pink and contain mostly spirilloxanthin and lycoxanthin; at higher concentrations (100 μg./100 ml.) the colonies are orange, containing about the same amount of lycoxanthin, much less spirilloxanthin, and considerable amounts of cryptoxanthin, a pigment not produced in low-thiamine media [Starr & Saperstein (124)]. Similar results were obtained



Formula IV. β-Ionone.



Formula V. Methylheptenenone.

with *Corynebacterium michiganese* mutants (78). In *Polytoma uvella,* thiamine is "a strong stimulant" for carotenogenesis and in *P. blakesleeanus,* although no qualitative variations occur, β-carotene production is somewhat inhibited at low thiamine levels (119). Grob *et al.* (125) found slight, but apparently significant, stimulation of β-carotene synthesis in *Mucor hiemalis* by pantothenic acid, pantethine, and possibly phosphorylated pantethine. According to Kizen *et al.* (126) lack of biotin stimulates pigmentation in *Torula* and *Sporobolomyces* spp., and Prickett & Massengale (127) found that ergosterol, but not irradiated ergosterol, increased pigmentation and growth in various *Mycobacterium* spp. When acetate is used as the sole carbon source, yeast extract stimulates growth and carotenogenesis in *P. blakesleeanus;* no such effect is observed when glucose is the carbon source. None of the known growth factors will replace yeast extract [Friend *et al.* (139)].

The ionone effect.—Mackinney *et al.* (128, 129) found that β-ionone (see Formula IV) added to germinated cultures of *P. blakesleeanus* considerably stimulates β-carotene synthesis. ψ-Ionone tends slightly to stimulate lycopene synthesis, while methyl heptenenone (see Formula V) and to a lesser extent citral, reduces β-carotene synthesis while stimulating that of phytofluene and ζ-carotene [Mackinney *et al.* (129, 130)]. In this respect methylheptenone resembles DPA (see below), and a further similarity is that its inhibitory action is also overcome by adenylic acid (AMP) [Goodwin &

Modi (unpublished observations)]. Tracer studies make it certain that the ionone effect is indirect in the sense that the compound is not incorporated *in toto* into β-carotene; the β-carotene produced is unlabelled when the β-ionone added is labelled either in position 9 [Mackinney *et al.* (130)] or 6 [Engel *et al.* (131)]. Further isotope studies will demonstrate whether the effect is completely indirect or whether the mold can split the ionone ring (at the dotted lines in IV) to produce the basic branched five C unit.

The β-ionone and methylheptenone effects are quite separate so that, using a 1:1 mixture, mycelia can be produced rich in both partly saturated polyenes and β-carotene [Mackinney *et al.* (132)]. The β-ionone effect, which is independent of light (66), is inhibited by streptomycin [Goodwin & Modi (unpublished observations)].

The diphenylamine (DPA) effect.—Specific inhibition of carotenogenesis by DPA with accompanying stimulation of the phytofluene group, first noted in *M. phlei* [Turian (133); Turian & Haxo (37)] and *P. blakesleeanus* (33) has now been reported in *Rhodospirillum rubrum* [Goodwin & Osman (43)], *Allomyces javanicus* (38), and *Chromatium* spp. (80). DPA has no specific effect on carotenoid synthesis in *E. gracilis* v. *bacillaris* (71) or *Chlorella vulgaris* (70) while in *Rhodopseudomonas spheroides* it inhibits growth considerably and pigmentation slightly for 3 to 4 days, then, quite suddenly growth and pigmentation become normal [Goodwin *et al.* (134)].

AMP^2, but not adenosine, overcomes the carotene-inhibitory action of DPA, but in no way affects its phytofluene-stimulatory power in *P. blakesleeanus*. Riboflavin is also similarly effective but, above a concentration of 1/200,000, itself inhibits carotenogenesis in normal cultures [Goodwin *et al.* (135)]. Neither adenylic acid nor riboflavin overcomes the DPA effect in *R. rubrum* (135). Washed DPA pads of *P. blakesleeanus* will synthesize β-carotene when placed on a glucose/salt medium, but there is no concomitant disappearance of the more saturated polyenes; no pigment synthesis occurs when the pads are placed on phosphate buffer alone (135). Washed DPA cultures of *M. phlei* will not regenerate pigment even on glucose (57), whilst washed DPA cells of *R. rubrum* will regenerate spirilloxanthin either anaerobically in the light, or aerobically in the dark, when merely resuspended in phosphate buffer. The saturated polyenes do not, however, disappear; bacteriochlorophyll is also regenerated simultaneously. Regeneration also occurs aerobically in the light but to a reduced extent; this correlates with reduced oxygen uptake by the cells in the light (43). No success has yet been obtained in regenerating spirilloxanthin in cell free extracts of DPA cultures, and it has also been found that the regeneration is very insensitive to the normal metabolic inhibitors, most of which completely inhibit growth [Goodwin & Land (unpublished observations)].

Turian (133) found Fe^{++} to reverse the DPA effect in *M. phlei;* later this could not be confirmed using a number of strains (including Turian's) of this organism (57) and did not occur in *P. blakesleeanus* (135).

Antibiotics.—On a glucose/asparagine medium, streptomycin has no effect

on growth of *P. blakesleeanus*, but it inhibits carotenogenesis linearly with increasing concentrations up to 0.01 per cent, when the carotene level is about 30 per cent of the normal value. Further increasing the concentration (up to 1.6 per cent) has no further inhibitory effect on carotene synthesis. When glucose is replaced by acetate, both growth and carotene synthesis are inhibited equally so that the carotene concentration remains normal [Goodwin & Griffiths (136); Schopfer *et al.* (137)]. When asparagine is replaced by NH_4NO_3 or $(NH_4)_2SO_4$, streptomycin inhibition does not occur; this is not a pH effect [Goodwin, Griffiths & Modi (unpublished observations)]. On a glucose/NH_4NO_3 medium, growth of *P. blakesleeanus* is less than on a glucose/asparagine medium, but can be raised to the level of the latter medium by addition of 0.1*M* succinate or acetate [Leonian & Lilly (138)]. Carotene synthesis is equally stimulated [Friend *et al.* (139)]; but while streptomycin has no effect in either case on growth, with succinate but not acetate, it inhibits carotene stimulation. Attempts to overcome streptomycin inhibition of carotenogenesis by pantothenic acid and hexosemonophosphate were unsuccessful [Goodwin, Griffiths & Modi (unpublished data)]. Streptomycin, although growth inhibitory, has only a slight specific effect on pigmentation in *R. rubrum* [Goodwin & Osman (140)]. The well known chloroplast destruction in *Euglena* spp. by streptomycin is accompanied by a considerable inhibition of carotenoid synthesis, although the pigments do not disappear as completely as does chlorophyll (71).

Chloramphenicol has a rather similar effect to streptomycin in *P. blakesleeanus* while penicillin up to a concentration of 0.02 per cent has no effect on either growth or carotenogenesis; chlortetracyline (aureomycin) has no specific effect on carotenogenesis although growth is considerably inhibited [Goodwin & Griffiths (unpublished observations)]. Heximide (actidione) inhibits carotenogenesis slightly in *P. blakesleeanus* [Goodwin & Modi (unpublished observations)], although it stimulates γ-carotene synthesis in *Allomyces arbuscula* [Whiffen (141)] and in *Blastocladiella* spp. [Cantino & Hyatt (142)].

Other inhibitors.—Turian (143) found phenol to be a potent inhibitor of carotenogenesis in *M. phlei*. This has been confirmed, but no saturated polyenes accumulate simultaneously (57). A number of well-known metabolic inhibitors had no specific inhibitory or stimulatory effect on carotenogenesis in *M. phlei* (57), these included 2,4-dinitrophenol, stated previously to stimulate carotenogenesis (144).

Genetical studies.—Four crosses from a wild type and albino *N. crassa*, *CI*, *Ci*, *cI* and *ci*, (*C* is the major colour gene and *I* the unlinked intensifier) were examined for carotenoids. Those from *CI* and *Ci* were qualitatively identical but quantitatively less than in the parent wild type, *ci* was without pigments and *cI* contained only traces. In *CI* light stimulated pigment production to a much greater extent than phytofluene production; this led to the conclusions that light is concerned with the conversion of the latter into pigments and that the gene *C* acts in the biosynthetic system before the production of phytofluene [Sheng & Sheng (144)].

An exhaustive study of a number of mutants of *N. crassa* allowed Haxo (39) to divide them into four main groups in which were eliminated: (*a*) the acidic pigments, (*b*) most xanthophylls, some carotenes but not phytoene, (*c*) all polyenes except phytoene, and (*d*) all polyenes including phytoene, As in the case of tomato crosses, exact interpretation of results is difficult but it appears that the acid pigments are final products of anabolism and that polyenes are more likely to be synthesized in parallel rather than sequentially.

A number of naturally-occurring mutants of *Phycomyces* all synthesized mainly β-carotene but differed slightly in the rates of synthesis [Goodwin & Griffiths (145)].

The carotenoids have recently been described in four x-ray mutants of *Chlorella vulgaris*. Three of the mutants will grow only in the dark on glucose: 5/871 synthesized phytoene only; 5/515 phytoene, phytofluene and ζ-carotene but no xanthophylls and 9a phytoene, phytofluene, ζ-carotene xanthophylls, and an unidentified carotenoid [λ_{max} (light petroleum), 404, 426, and 452 mμ)]. In each case the normal β-carotene has been replaced by members of the phytofluene series, the effect of mutation being very similar to the action of DPA. Strain 5/520 is most interesting in that it will grow in the light and produce chlorophyll and the "normal" carotenoids (β-carotene and xanthophylls), while in the dark it produces none of these pigments but phytoene, phytofluene, ζ-carotene, and a number of unidentified pigments probably including tetrahydrolycopene and lycopene. Preliminary experiments suggest that on bringing a dark culture into the light, the phytofluene series disappears as β-carotene is synthesized (123).

General cultural studies.—P. blakesleeanus grows sparsely on a medium containing lactate as the sole carbon source and contains little or no carotene; addition of acetate results in stimulated growth and carotenogenesis [Schopfer & Grob (146)]; later it was shown that acetate alone in the presence of NH_4NO_3 was sufficient to support growth and carotene production (Schopfer & Grob (147)], the optimal acetate concentration being 0.5 per cent (139).

Stimulation of carotenogenesis in *P. blakesleeanus* by replacing asparagine in a standard medium by leucine and valine [Goodwin & Lijinsky (148)] has been confirmed in another strain, but in this case the effect is also observed in a 3 per cent glucose medium [Chichester *et al.* (149)], whereas in the original experiments the glucose level had to be reduced to 1 per cent before the effect became pronounced. The stimulation was considered attributable to the liberation of the branched 5 C unit from the amino acids, in the case of leucine after the decarboxylation of the α-ketoacid. Isotope experiments show that 1-C^{14}-leucine is quickly decarboxylated by *P. blakesleeanus* and that only little activity appears in the carotene; with 2-C^{14}-leucine, on the other hand, considerably more activity appears in the carotene, but it is not sufficient to account for all the increased production of carotene (149). Thus the mechanism appears more complicated than the direct *in toto* incorporation into carotene of the branched 5 C-unit from leucine, although this must

occur to some extent. Experiments using possible intermediates in the conversion of leucine to a 5 C-unit have not yielded clear-cut results in either *P. blakesleeanus* [Goodwin *et al.* (150)] or *N. crassa* (120), although in the former some stimulation was observed with β-methylcrotonaldehyde; the main reason is probably that the intermediates are presented to the moulds in an inactive form at one time and in small concentrations (they are all somewhat toxic), while the metabolism of leucine provides a constant flow of active intermediates. Leucine and valine have only a slight stimulatory effect on carotenogenesis in *R. rubrum* and then only in aerobic cultures (140) and no effect in *C. poinsettiae* (124) or *Sarcina lutea* [Arkani & Stary (151)]. Leucine but not valine is stimulatory for *Rhodotorula shibatana* (Ishii (152)].

Of a number of carbon and nitrogen compounds tested, only pyruvate, aspartate, and glycine unequivocally stimulated carotenogenesis in *S. lutea:* alanine, oddly enough, was inhibitory (151).

An obstacle in studying red yeasts has been the difficulty in liberating and extracting the pigments quantitatively, the usual method of heating with 0.5 *N* HCl for 15 min. at 100°C. being somewhat drastic and not infallible. Important analytically and interesting metabolically is the observation that when grown on Wickerham's medium in shake cultures the pigments can be extracted from *Rhodotorula* spp. with cold acetone [Peterson *et al.* (153)]. The problem of extraction from other media remains. Culturing under high pressure which allows easier extraction of carotenoids from some marine microorganisms has no similar effect on *Rhodotorula* spp. [Courington & Goodwin (Unpublished observations)]. An analytical difficulty has also been revealed in studying marine bacteria containing polyhydroxycarotenoids; the usual technique of grinding with anhydrous Na_2SO_4 and then extracting with a fat solvent cannot be used either because these xanthophylls are adsorbed very strongly on the Na_2SO_4 or because drying with this reagent does not break down the protein-pigment complex; in such cases the pigments are extracted by warming the wet cells with ethanol (56). A very effective preliminary to extracting pigments from marine diatoms is to disrupt the cells by placing them in distilled water [Baalsrud (personal communication)].

Glycerol is reported more effective than glucose for growth and carotenogenesis in *R. shibatana* (152), and this is also true for carotenogenesis but not growth in *M. phlei* (57).

Tryptophan, asparagine, arginine, histidine, and leucine were all stimulatory in *R. shibatana* (152). Attractive colour photographs showing the effect of various natural media on pigmentation in a number of yeasts have been published by Etchells *et al.* (154).

A mutant strain of the water mould *Blastocladiella* synthesizes mainly γ-carotene, while the parent strain produces no carotenoids. Furthermore, the mutant lacks α-ketoglutarate oxidase which is present in the parent (74). The temptation is great to suggest a causal relationship between these two observations and to conclude that when the tricarboxylic acid (TCA) cycle is blocked metabolites are funnelled into carotene synthesis. Support for this comes from the observation that when the TCA cycle is inhibited by HCO'_3

in the wild strain, it synthesizes γ-carotene [Cantino (personal communication)].

Confirmation has been obtained of the observation that *Rhodopseudomonas spheroides* produces mainly a yellow carotenoid (Y) when grown anaerobically and that when anaerobic cultures are allowed access to O_2, a red pigment (R) is produced in amounts almost equal to the amount of Y disappearing. Resting cells also undergo this change when suspended in buffer only, and a number of metabolic inhibitors which prevent growth of the organism do not inhibit this pigment change (134).

If asparagine or ammonium acetate replaces glycine as the N source in the culture medium, *Polytoma uvella* synthesizes colorless fluorescent polyenes different from phytofluene (24).

Haematococcus pluvialis cultured on either a soil extract or ammonium acetate/KNO_3 medium, synthesizes β-carotene, lutein, and neoxanthin when in the green stage; after about 10 days it encysts (reddens) and produces large amounts of astaxanthin, but not at the expense of the β-carotene etc. which remain at the same concentration. No obvious polyene precursor of astaxanthin was present in the nonencysted cells and no metabolic inhibitor which did not kill the cells could be found to prevent the synthesis of astaxanthin in washed green cells (72). Light is not essential for astaxanthin synthesis but considerably accelerates the process [Droop (155, 156)]. Examining the effect of various N sources on astaxanthin synthesis in *H. pluvialis*, Wurtz (157) found that with asparagine, alanine, or NH_4NO_3 no pigment was formed even after five weeks; with glycine very slight formation occurred after six weeks, but the cultures were in poor shape; addition of sodium acetate to the glycine medium stimulated astaxanthin production within eight days. Considerable pigment was also formed within three weeks on leucine, arginine, threonine, proline, peptone, valine, creatine, taurine, sarcosine, alanylalanine, and glycylalanine. On methionine, lysine, phenylalanine, tyrosine, and ammonium acetate growth was poor but cultures were red from the beginning. No growth occurs on asparatate, glutamate, β-alanine, serine, chloracetyl-leucine, histidine, betaine, KNO_3 (alone), $NaNO_2$, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, or ammonium formate. Droop (156) has confirmed that astaxanthin synthesis is independent of available nitrogen but not of carbon supply; furthermore, astaxanthin is not synthesized in proliferating cultures even under otherwise favourable conditions. Astaxanthin will disappear from cells which begin to multiply.

Isotope studies.—Apart from investigations already discussed previously in this review, it has been shown that on an acetate medium, *P. blakesleeanus* incorporates the carboxyl and methyl group of acetate into carotene to about the same extent [Grob *et al.* (158)]. Using the same medium, Grob & Bütler (159, 160) have also demonstrated that the lateral methyls of β-carotene arise from the methyl of acetate, as do the methyls at positions 5 and 5′; the C atoms adjacent to all these methyls arise from the carboxyl C of acetate. In the presence of glucose, Glover *et al.* (161) could find no specific incorporation of acetate into carotene by vegetative cells of *P. blakesleeanus*.

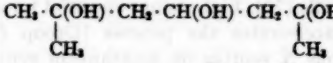
Discussion

It is apparent that a great deal of ground work has cleared away a number of antediluvian concepts concerning carotenogenesis, but information at the substrate level remains exiguous. Carotenoids share with steroids, terpenes, and rubber, a common branched 5-C building unit—the isoprene residue. From information available on these various substances, it appears that carotenoids are probably synthesized from an active 2C unit. In the Coenzyme A form, the following reactions can take place:

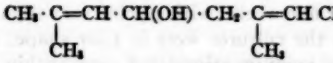
$$\underset{\text{acetate}}{\overset{\text{I}}{\longrightarrow}}\ \underset{\text{acetoacetate}}{\overset{\text{II}}{\longrightarrow}}\ \underset{\beta\text{-hydroxy-}\beta\text{-methylglutarate}}{\overset{\text{III}}{\longrightarrow}}\ \underset{\beta\text{-hydroxy-}\textit{iso}\text{valerate}}{\overset{\text{IV}}{\longrightarrow}}\ \underset{\beta\text{-methylcrotonate.}}{}$$

Reactions I→III occur in flax seeds [Millerd & Bonner (162); Bonner *et al.* (163)] but IV has not yet been demonstrated in plant tissue although it does occur in liver [see e.g. Rudney (164)].

Either β-hydroxy*iso*valerate or β-methylcrotonate could polymerise to produce a C_{40} carotenoid precursor, the former giving a type compound illustrated in formula VI and the latter a compound of formula VII.



Formula VI



Formula VII

In neither scheme should methyl and carboxyl labelled acetate give rise to the same degree of labelling because of the decarboxylation in reaction III. In the experiments from which it was concluded that this labelling was the same the scatter was considerable (158) and the conclusion perhaps unjustified. Furthermore, neither the phytofluene series nor materials of low adsorptive power with absorption spectra in the ultraviolet region of the spectrum would be precursors. (The hydroxyl groups would increase the adsorptive power and the complete absence of double bonds or their presence unconjugated would preclude ultraviolet absorption.) A C_{40} compound based on VI might even be water soluble. The failure to detect coloured intermediates in the regeneration of spirilloxanthin in DPA cells of *R. rubrum* (43) and in the synthesis of astaxanthin in *H. pluvialis* (72) may be a result of the simultaneous removal of all the water molecules from a C_{40} precursor.

These suggested pathways may be incorrect; but they will serve their purpose if they direct attention away from the concept of the partly saturated polyene precursor, which, at the moment appears to have reached its limit of fruitfulness. Other theoretical aspects of carotenogenesis have recently been reviewed [Goodwin (165)].

It will be salutary to end this section by indicating, as questions, some of the deficiencies in our knowledge of carotenogenesis which will remain even if the mechanisms suggested above were proved to occur: (*a*) how does the tail-tail condensation of the 5 C units at the centre of all carotenoid molecules take place and, furthermore, is the first step in synthesis a tail-tail condensation followed by head-tail condensations at either end of this primary 10 C unit or the production of a 20 C unit which then condenses tail-tail with itself; (*b*) how do carbons 2 and 2′ in β-carotene (I) become saturated; (*c*) how do isomers such as α-, β-, and γ-carotenes arise; (*d*) when and how is oxygen incorporated into xanthophylls; and (*e*) what determines the synthesis of *cis*- and *trans*-isomers?

FUNCTION IN PLANTS

The best authenticated function of carotenoids in plants is in transferring light energy to chlorophyll for photosynthesis. This has recently been reviewed in detail [Blinks (166)] and will not be considered here. A possible major role in reproduction, suggested by much circumstantial evidence [see e.g. Goodwin (167)], is becoming less likely with the appearance of recent information. For example, (*a*) a sunflower mutant completely carotenoid-deficient sets viable seeds to its own colorless pollen (98); (*b*) with γ-carotene deficient (DPA) gametangia of *Allomyces javanicus*, formation of planozygotes is not prevented although motility of the male gametes was somewhat reduced [Turian (168)]. It should be noted, however, that there are probably always traces of γ-carotene present in DPA cultures and the more saturated polyenes are present in increased amounts (138); it might, therefore, be concluded either that the trace of γ-carotene is sufficient for reproductive activity or that the more saturated polyenes can replace γ-carotene in this function. No direct stoichiometric relationship exists between β-carotene synthesis and zygote formation in *P. blakesleeanus*, although, in one case, when β-carotene synthesis is reduced by buffering the medium to pH 7.0, zygote formation is also inhibited. There is, however, little accompanying effect on formation of gametangia and suspensors. If, therefore, there is a relationship between carotenoids and sex in *P. blakesleeanus*, these experiments suggest that the pigments are associated with the early stages of zygote formation [Burnett (169)].

ANIMAL METABOLISM

Space available does not allow discussion of this important branch of carotenoid biochemistry, but some aspects, especially the problem of the factors controlling conversion of carotene into vitamin A have recently been reviewed elsewhere [Goodwin (170)].

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NUTRITION[1]

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The purpose of this review is to draw attention to certain growing points and trends in clinical nutrition which may be of interest to nutritional biochemists. It is essentially eclectic in relation to the author's own outlook as a physician interested in the clinical aspects of undernutrition and malnutrition in a country where comparisons of different racial groups at various stages of cultural and economic development is possible. It concentrates particularly on the African continent and its comparison with other parts of the underdeveloped world.

Throughout the African continent recent work, particularly arising out of interest in the disease kwashiorkor, suggests that protein malnutrition may be of equal importance with tropical parasitic disease and enervating climates in determining a high incidence of disease and backwardness in cultural and economic development. In this vast region population levels are low in relation to area when compared with many unprivileged parts of the world, such as the Far East. Control of tropical diseases is advancing rapidly through tropical hygiene and the appearance of antibiotics and chemotherapeutic weapons against tropical parasites. As a result of the control of tropical parasitic disease there are indications that the population may increase very rapidly and yet even with the present population there seems to be difficulty in providing calories, and there is every evidence that protein foodstuffs are lamentably deficient.

Although outside the scope of this review it should be noted that recent studies of blood groups, the sickling phenomenon, and the distribution of the varieties of hemoglobin are beginning to throw a fascinating light on the physical anthropology of the continent and will undoubtedly furnish clues to genetic factors underlying susceptibility and resistance to disease. The relationship between the sickling phenomenon and resistance to malaria is the most obvious example (1).

In the Union of South Africa there is a special opportunity for study of some of these factors. In the first place all but a small area of the Union is free from strictly tropical diseases such as malaria and trypanosomiasis, while the pattern of dietary protein deficiency in the numerically predominant Bantu people is similar to that of Bantu and Negro people through the tropical belt of the Continent. Among the Bantu people of the Union of South Africa food intake is determined by traditional, social and tribal cus-

[1] The survey of the literature pertaining to this review was completed on September 30, 1954.

tom. Calories are derived largely from maize (corn), large herds of scrub cattle are kept on overgrazed land and regarded as a measure of wealth and for the bride price (lobola). Quantity is preferred to quality in cattle and milk yields are pathetically low. Because of the social importance of large numbers of cattle to chiefs and head-men, meat is consumed only occasionally on feast days. Soured milk is the principal source of animal protein. Rural diets consist therefore very largely of home pounded maize, soured milk, and some naturally growing vegetables. Migrant Bantu labourers who go to the cities and those who become partly or permanently urbanised have their traditional dietary patterns upset without understanding the nutritional value of foodstuffs available in the cities. Home pounded maize is often replaced by degerminated and purified mazie meal and later tends to be replaced by white bread. All protective foodstuffs have to be purchased from the limited cash which it is his object to take back to his rural community if he is a migrant labourer. In highly organised industries such as the gold mining industry, however, the Bantu labourer is fed a generous and scientifically planned diet and after a period of time he may be among the best fed people in the country.

Interracial comparisons in the vast and extremely complicated racial problems of South Africa constitute a unique opportunity for observation on the part of those who are interested in the effects of environment on public health and in particular in the effects of diet on health and disease. The National Nutrition Council of South Africa has published some interesting and progressive reports and recently a Ministry of Nutrition and a National Institute for Nutrition Research have been set up. To meet the problems of protein malnutrition and the deleterious results of purification of cereals experiments are being made in the fortification of wheat and maize flour with vitamins and with protein drawn from skimmed milk, soya bean, and fish meal. Staple foodstuffs are extensively subsidised. Great efforts are being made to persuade industrialists to provide to labourers one free meal per day containing generous quantities of protective foodstuffs.

In Johannesburg with its long established gold industry and rapidly developing heavy industry migrant Bantu labourers are drawn from many Bantu races and from many areas in Southern Africa, both tropical and non-tropical. It is against this background that many studies have been made of the relationship of malnutrition to disease in Bantu people and the relationship between cirrhosis of the liver and hepatoma. The scope of this work is represented by two books, *Perspectives in Human Malnutrition* by Gillman & Gillman (2) and *Primary Carcinoma of the Liver* by Berman (3).

In the writer's own area another opportunity for comparative study of the effects of nutrition is provided by the coexistence of European and Cape Coloured people in the neighbourhood of the city of Cape Town. The Cape Coloured people are a fairly homogenous race derived from an earlier racial intermixture of the original Hottentot inhabitants of the southern end of the continent, Malay slaves introduced by the Dutch East Indies Company

from the Far East, European blood, and recently a small amount of Bantu blood. Their pattern of health and disease has been described by Brock (4). They eat according to European dietary patterns, but the majority are economically underprivileged. Until comparatively recent years the Cape Province of which Cape Town is the capital, had a comparatively small Bantu population except in the northeast region which is known as the Transkei Reserve. In recent years, however, there has been increasing migration of temporary Bantu labour from the Transkei Reserve to Cape Town and surrounding areas. In this group it is possible to study the effects of life-long protein malnutrition with reasonable certainty of the absence of complicating results from true tropical parasites. Superimposed on this chronic ill-health by the conditions of migrant labour there appear well recognised severe vitamin deficiency states, such as scurvy and thiamine deficiency.

Before entering into the problems of undernutrition and malnutrition which arise out of these introductory remarks attention is drawn to an interesting shift in emphasis of nutrition research in privileged countries and exemplified by the proceedings of the Third International Nutrition Congress at Amsterdam in September, 1954 (27). This shift in emphasis is towards the study of overnutrition and of the possible adverse effects of intentional food additives.

Overnutrition

A growing recognition in medicine of the statistical association of many diseases with obesity has led to more critical appraisal of calorie requirements. The 1953 recommended dietary allowances of the American Food and Nutrition Board (5) has followed the lead of the Food and Agriculture Organisation of the United Nations (6), in reducing the standard man and woman from 70 kg. and 65 kg. to 60 kg. and 55 kg. respectively, and in making reductions for increasing age (5 per cent per decade over age 25) and for higher than average mean annual temperature.

This trend was recognised at the Nutrition Congress by setting the first day of the Congress aside for a discussion on "overnutrition and disease." The discussion included papers on obesity and life expectancy, standard body weight, and the estimation of the body fat compartment by measurement of skin-fold thickness. More important, however, was a series of papers on the mechanism of obesity. Central among these was the paper by Mayer on mechanisms of regulation of food intake and multiple etiology of obesity. In this paper new evidence was put forward confirming and extending the theory of the glucostatic mechanism of regulation of food intake (7). According to this theory the arteriovenous glucose difference (delta-glucose) is critical in controlling hunger feelings and gastric contraction. Psychological stimuli succeed in evoking hunger contractions in the stomach only if delta-glucose is less than the limiting value of approximately 8 mg. per cent for the finger blood-antecubital vein difference. This is now shown to apply under a variety of experimental situations including calorically adequate diet,

calorically insufficient diet, diabetes mellitus, cortisone treatment, and obesity. The complexity of the obesity mechanisms was illustrated through three different types of experimental obesity in mice: (*a*) genetic (hereditary obese-hyperglycemic syndrome), (*b*) gold thioglucose induced obesity, and (*c*) hypothalamic obesity. These three types of obesity have been studied in litter mate mice, and many of the endocrine and chemical characteristics of the three types have been compared.

One aspect of overnutrition has occupied great interest for the last few years, namely, the possible relationships between atherosclerosis, serum cholesterol, and diet.

Coronary heart disease, serum cholesterol, and diet.—This subject has received intensive study and report from many parts of the world. It cannot be reviewed in full here, but a few points are worth making. The general trend of observation and opinion may be summarised as follows: There appears to be a rising incidence of coronary heart disease in recent years, with a trend to occurrence in younger age groups; there is a marked sex disproportion so that women are relatively immune at least up to the menopause. The incidence appears to be greatest among the more economically privileged nations of the world and among the more economically privileged groups within a nation. These trends in the male sex have been attributed to four main groups of factors: (*a*) tension and strain of modern life, (*b*) lack of physical exercise, (*c*) cigarette smoking, and (*d*) alterations in dietary intake pattern. Only the last of these four alleged factors is relevant to the present discussion, but it cannot be considered except against the background of the other three. The dietary theory of etiology alleges that the rising incidence of coronary heart disease is linked with the effect of changing dietary patterns on the level of cholesterol or lipoproteins in the blood. Of these blood fractions the total cholesterol at least is admitted normally to be higher in the male than in the female and to increase with age, but for a given sex and age group it is claimed that modern dietary trends are leading to higher average levels. This is held to account for the increasing incidence and lower age of occurrence of coronary heart disease.

Before considering the nature of the alleged modern dietary trends which might influence coronary heart disease, it is necessary to consider certain differences in interpretation. In general it may be said that, in countries where vital statistics are available and medical services highly developed, the assessed incidence of coronary thrombosis is likely to be reasonably accurate. In underprivileged populations, on the other hand, a reasonably accurate figure for the incidence can probably only be attained through post-mortem records, which often apply to highly selected fractions of the total population.

It will probably be generally agreed that most coronary thrombosis occurs on a basis of coronary atheroma or atherosclerosis, but for a given degree of coronary sclerosis there may be many humoral and other tissue factors which determine whether thrombosis occurs or not. There is some evidence that some of the humoral factors (e.g., estrogens and heparin) may control

not only *in vivo* coagulation but possibly, also probably more slowly, the development of atheroma. It must be concluded, therefore, that trends in mortality from coronary thrombosis such as those described by Strom & Jensen (59) may arise either from changes in the incidence and severity of atheroma or changes in the tendency for *in vivo* thrombosis. Either of these changes might be affected by diet.

There has been considerable discussion of whether the alleged effect of modern dietary trends on coronary heart disease is affected through the level of total cholesterol of the blood or through other fractions of cholesterol or lipoproteins, or through Gofman's macromolecules as estimated by flotation techniques (53, 54). Many other humoral factors will need to be considered before the problem is settled.

In spite of many uncertainties there appears to be a valid statistical correlation between the incidence of coronary heart disease in men at a given age and higher-than-average levels of cholesterol or lipoproteins for that age group. This relationship has been most convincingly demonstrated so far for total blood cholesterol (55). Finally, the nature of the relationship between diet and blood colesterol for a given age group in men must be discussed. The strongest evidence so far presented links the upward trend of blood colesterol and of coronary heart disease in men with a rising fat/calorie ratio in the diet, that is with a rising percentage of total calories derived from dietary fat. The correlation is convincingly close, but the statistical association has not yet been proved to be causative. Modern privileged high fat diets differ in many other respects from primitive and underprivileged diets. Three differences, among many others, which have been considered are cholesterol content, crude fibre content, and the proportion of fat drawn respectively from animal and vegetable sources. All of these need to be seriously considered along with the more simple fat/calorie ratio. Whatever the mechanism may be of the effect of changing dietary patterns on blood chemistry and of blood chemistry on the development of atherosclerosis, there can be no doubt that a *prima facie* case has been presented for investigation. At the same time nondietary factors such as exercise (8), cigarette smoking (57), and emotional tension can be allowed a contributory role.

In spite of the inadequacy of vital statistics there is a general impression that death from coronary heart disease is uncommon in many primitive and underprivileged communities. This is almost certainly true for the Bantu of Southern Africa (9), but it is interesting that there is a widespread impression that in other parts of the body Monckeberg's calcific arteriosclerosis is not at all uncommon. Evidence on the level of blood cholesterol at different ages in Bantu subjects, is, therefore, welcome (10). It shows in males a distinctly lower level than in Americans for each age group.

Intentional Food Additives

Another very significant trend in this International Nutrition Congress was the attention devoted to "non-nutrient chemical substances in foods (intentional additives)." It is clear that with the increasing urbanisation of

populations and necessity for greater international trade in food, methods of canning and preservation of foods will become increasingly important and there will be greater need for the addition of preservatives. In addition, there has been great development in the last decade of materials added to processed foodstuffs to improve their texture and appearance. In the introductory paper Bacharach pointed out that any distinction between synthetic and natural additives is, to the chemist, unjustifiable, and there is no difference in principle involved in deciding on the addition of *p*-aminobenzoic acid as a vitamin or *p*-hydroxybenzoic ester as a fungicide. A plea was then made by J. M. Barnes for a more widespread use of subacute toxicity tests and a criticism of long-term feeding tests in routine use. An extremely interesting paper was given by H. Druckrey (27) on dyestuff food additives. He divided harmful additives into those with a threshold effect in which any damage done by subtoxic doses is quickly and completely reversible and, on the other hand, those with a summation action in which the sum of all doses given over a long period of time might produce an irreversible effect. The latter group are well represented by the carcinogens. In connection with the carcinogenic action of azo-dyes, he pointed out that only a limited number of representatives of this group are toxic and that it is wrong to conclude that all fat-soluble azo-dyes are carcinogenic. He made the point further that carcinogenic action may appear in a heterogenous group of dyestuffs and that the mere change of a methyl group may render harmless substances carcinogenic. Only a few dyestuffs are carcinogenic, and many carcinogens are colourless. It was clear from the communications and discussion that although there was nothing inherently wrong in the incorporation of intentional additives in processed foodstuffs, very great care would have to be exercised in their selection and testing if adverse effects on health were to be avoided. This appears to be a sphere ripe for international agreement.

Antibiotics in Relation to Nutrition

A very interesting review was given at the Nutrition Congress by D. Catron (27) of the role of antibiotics in animal nutrition, the economic significance of which in the meat and poultry industries is now very significant. It was stated, for example, that in the pig rearing industry antibiotics may save as much as 20 per cent of feed per 1 lb. gain in live weight. Most important economically is its sparing effect on protein intake, but in addition there is economy in riboflavin, niacin, pantothenic acid, and probably other vitamins. One of the most interesting aspects reported is the unequal effect on different litter mates tending to encourage the runts and to smooth the whole litter out to a more uniform growth and development. This is, of course, consistent with variations in the intestinal flora of litter mates. The effect on pigs is partly in the control of swine enteritis as is shown by experiments in which swine are "challenged" by oral innoculation of the causative organism. A similar effect may be exerted on other epidemic diseases of animals. It is certain, however, that this does not account for

all the growth promoting effect of the antibiotic. There is undoubtedly also an effect exerted through the action of the antibiotic on the "normal" intestinal flora. This conclusion is implicit in most of the work in this field and particularly obvious under strictly controlled conditions as in the germ-free life laboratory of the Lobund Institute (11).

Before considering the mode of action of antibiotics on intestinal flora it is well to recall some history. Knowledge in this field of mammalian nutrition probably began with the demonstration of the phenomenon of refection by Fridericia in 1926 (56) and defined as "the ability, observed in a certain proportion of rats, to develop the capacity to grow normally on a vitamin B-free diet." Modern microbiology has made an intensive study of the nutrition of parasites, bacteria, and viruses, all of which relates to mammalian nutrition, since symbiosis and antibiosis in the mammalian intestine depend to a considerable extent upon available food supplies for the microorganisms. At the same time the mammal under healthy conditions can be regarded as living in a state of symbiosis with his intestinal flora. Since the demonstration of the phenomenon of refection, it is apparent that mammals may be dependent on their commensal intestinal flora at least for digestion of cellulose, synthesis of vitamins, and protection from invasion of the gastrointestinal tract by pathogenic microorganisms. These principles presumably all apply to man as a mammal.

Further, it should be recalled that even before the introduction of antibiotics the use of sulpha drugs had demonstrated the role of normal intestinal bacteria in synthesising folacin and biotin in the nutrition of the rat.

Some aspects of the mode of action of antibiotics in promoting growth have recently been reviewed (12, 13, 14, 17).

The contribution of the normal commensal bacterial flora of the mammalian and avian gastrointestinal tract to the nutrition of the host and the effect on these organisms of antibiotics is evidently complex. The variability of growth and development in normal litter mates and the smoothing-out effect referred to by Catron (*loc. cit.*) constitutes a warning that results obtained even on litter mates are not necessarily applicable to other litter mates. The results of antibiotic feeding are obviously applicable only to precisely defined conditions which include the animal species, the geographical and climatic situation, colony hygiene, the occurrence of epidemics, and the exact constitution of the diet.

A recent observation is the effect of alteration of the intestinal flora by antibiotics on appetite. When penicillin is administered to growing chicks it is found that the growth-promoting effect is dependent on increased intake, and presumably appetite. The growth-promoting effect is not seen if the intake is artificially maintained at a level not higher than the intake of the control chicks. Again in the case of penicillin administered to chicks, the increased intake and growth promotion appears to be positively correlated with the *Bacillus coli* count and negatively correlated with enterococci in the faeces (15). It is clear from a number of studies that alteration in the balance

of commensal organisms in the gastrointestinal tract may exert a growth-promoting effect in at least three separate ways: (*a*) the effect on appetite referred to above, (*b*) destruction of organisms which compete with the body for certain essential nutrients, and (*c*) destruction of organisms which synthesise nutrients required by the body. Gyorgy (17) suggests two other possibilities, namely, the production of metabolites by the intestinal flora which are then absorbed with toxic action on the host, or even the prevention of invasion of the liver by organisms from the intestine. It seems unlikely that the antibiotics which have a growth-promoting effect all have in common a chemical factor which after absorption has a direct growth-promoting effect on the host, but this possibility has not yet been completely excluded. Garrod (16) suggests yet another possibility, namely, that the antibiotic may stimulate certain microorganisms which may have adverse effects. He draws attention to the evidence that subinhibitory concentrations of various antibacterial substances, including antibiotics, can accelerate bacterial growth.

From the practical point of view a very important observation was made by Catron in his Iowa experiments. There was no evidence over a five-year period of loss of antibiotic effect by emergence of antibiotic-resistant strains of organisms. Gyorgy (17), on the other hand, records clear evidence of the alteration of nutritional effect produced by the action of antibiotics in the gastrointestinal tract as antibiotic-resistant strains emerge, a trend which is apparent in the work of other people as well.

From Africa comes another interesting facet of the effect of antibiotics on intestinal flora. Foy & Kondi (18) follow up previous publications demonstrating an excellent response to oral or intramuscular penicillin in some cases of megaloblastic anaemia studied in Kenya. In discussing their results they conclude that even the action of intramuscular penicillin is probably dependent on alteration of the intestinal flora, and that the response to penicillin is in some way associated with the production, utilisation and absorption of vitamin B_{12} in the intestinal tract. They suggest, further, that "the high incidence of megaloblastic anemias in many backward countries may be connected with the high carbohydrate low protein diet that produces an intestinal environment inimical to the synthesis and utilisation of B_{12}."

Bifidus Factor in Milk

Gyorgy (17, 27) in presenting at the Nutrition Congress another chapter on this subject summarised the position as follows. A strain of *Lactobacillus bifidus* was recovered from the faeces of young infants at the breast. This strain shows scanty growth or no growth on the semisynthetic media suitable for ordinary strains of *L. bifidus*. This variant strain has been named *L. bifidus* var. Penn. It is present in colostrum and milk from many mammalian species but is relatively low in the milk of ruminants. For this reason human milk is much richer than cow's milk. The factor has not been identified, but it appears that it belongs to the group of N-containing polysaccharides with high hexosamine content. There are resemblances between the activity of

bifidus factor in biological secretions on the one hand and blood group activity and intrinsic factor on the other hand. These resemblances foreshadow interesting associations.

Protein Foodstuffs

The work of the last half-decade has concentrated attention on the importance of protein foodstuffs in nutrition and in the prevention of ill-health. It is evident that such attention will help to elucidate the etiology of many human disorders affecting almost every system of the body. Interest in the world wide prevalence of kwashiorkor as an expression of deficiency of protein foodstuffs in the diet of the postweaning child has become a focal point in a new forward movement in nutritional science, partly because of the recent recognition of its enormous public health importance, but partly also because development of new knowledge of this human syndrome of malnutrition happened to coincide with a big advance in experimental knowledge of the effects of protein deficient diets on the integrity of the liver. The overlapping of nutritional interests in the human field and in the animal experimental field is emphasised by the report of the symposium on nutritional factors and liver diseases sponsored by the New York Academy of Sciences (24). The public health interest in Africa is emphasised by the reports of the Third Session of the Joint Food and Agriculture Organization, World Health Organization Expert Committee on Nutrition (29) and the Report of the Second Inter-African Conference on Nutrition held under the Auspices of the Commission for Technical Co-operation in Africa South of the Sahara (C.C.T.A.) (30). Interest in the same trends in other parts of the world appears from the Report of the Third Meeting of the Nutrition Committee for South and East Asia (31) and the Report of the Third Conference on Nutrition Problems in Latin America (32).

The term "protein foodstuffs" is deliberately used here in leading up to a discussion of "protein malnutrition" since the latter term has been misunderstood and misquoted in some quarters as implying an exaggerated stress on the nutritional role of protein in the chemical sense of the term. It must be emphasised here that (*a*) each protein food differs not only in its amino acid composition but also in the variety and quantity of other nutrients, including vitamins and minerals, and (*b*) that at least in all the underprivileged parts of the world deficiency of protein foodstuffs in the diet means deficiency not only of amino acids but of other nutrients and especially of vitamins. It is worthwhile to stress the close association in nature of the vitamin B complex with protein foodstuffs and the interrelationships which exist between the metabolism of amino acids and of vitamins, particularly those of the B group. The interrelationship of tryptophan and niacin is only one example of this close association. The importance of trace elements in relation to vitamin and amino acid metabolism is evident in the place of cobalt in vitamin B_{12} (cyanocobalamin) and of molybdenum (25) in the xanthine oxidase factor.

Protein Malnutrition

The great attention which has been paid in the last few years to the study of kwashiorkor (19, 33) has emphasised the part played in the malnutrition of a large part of the underprivileged world by the use of starchy paps as the staple foodstuffs for the infant after it has been weaned from the maternal breast and also during the latter part of breast-feeding. In the second year of lactation the quantitative yield of protein must be grossly inadequate for the needs of the growing child. At this stage of life the mother's nipple may be little more than a dummy or comforter to the infant. The breast may yield small quantities of essential amino acids and vitamins or perhaps unknown accessory food factors, but it cannot supply either the calorie or the amino acid needs of the growing infant. Consideration of these facts led the Joint Food and Agricultural Organization, World Health Organization Expert Committee on Nutrition (29) to define the concept of "protein malnutrition" in weanlings in the following terms.

> In the field of medicine, public health and medical research, attention has recently shifted from disease due to deficiency of vitamins and minerals to what can provisionally be called protein malnutrition. This term cannot at present be defined within narrow limits. It is used here to indicate in general a state of ill-health occurring where diets are habitually poor in protein, while they are more nearly adequate in calories. Clinically, protein malnutrition is most easily recognised when there is a relatively high intake of calories from starchy foods together with an insufficient intake of protein. The concept includes the effects of deficiency in the quantity of protein consumed, of imbalance of amino acids, and of deficiency of factors such as vitamin B_{12}, commonly found in foods in association with animal protein and concerned with protein metabolism.

In some quarters this definition has been misconstrued, and it has been suggested that those who believe that kwashiorkor is attributable to "protein malnutrition" are thinking exclusively in terms of amino acids and do not recognise the part which may be played by known vitamins or even unknown accessory food factors including trace elements. This criticism which has been widely made is represented by the remarks of Schwarz (34). The misconstruction is quite unnecessary if the definition of the Joint Committee is carefully read. Although that definition did not directly mention unidentified accessory food factors, the example quoted of vitamin B_{12} was intended to indicate that other accessory food factors might still be discovered to have important interrelationships with the metabolism of amino acids. With well-known vitamins such as niacin the part played in the genesis of certain aspects of "the syndrome of kwashiorkor as an expression of protein malnutrition" was clearly recognised. At the C.C.T.A. meeting in Gambia, West Africa in November, 1952 (30) and at the Jamaican Conference on Protein Malnutrition (26) evidence was brought forward that in areas where the starchy pap was prepared from maize (corn) the dermatosis might be pellagrous in character while in areas where the starchy pap was prepared

from other cereals (e.g., millet) or bananas or roots there might be little or no dermatosis, and what there was might have no resemblance to pellagra. It is clear that "infantile pellagra" is kwashiorkor plus a pellagrous dermatosis and that the dermatosis might even be correctable in part by administration of niacin without correcting the underlying disease. The latter would still be fatal if protein in better quantity or quality were not introduced into the diet.

It is the author's belief that the introduction and popularisation of the term "protein malnutrition" has been healthy in the fields of medicine, public health, and medical research. It is steadily becoming clear that deficiency of protein foodstuffs may constitute the limiting factor in world nutrition and that attempts to satisfy the pangs of hunger by the development of crops such as cassava (manioc) with very high calorie yields per acre may merely shift the emphasis from starvation and undernutrition to protein malnutrition with its serious and disabling sequelae. The limiting deficiency in protein malnutrition is likely to be deficiency of certain amino acids but that does not exclude the possibility that vitamins and allied accessory food factors commonly found in association with animal protein may play an important part in determining the pattern of clinical nutrition and even the minimum or optimum requirement of individual amino acids. The use of the term is still consistent with the stress laid by Gillman & Gilbert (35) on "the constellation of factors governing the integrity of the liver and of other tissues." In reply to the criticism of these authors it must be stated that Brock & Autret (33) did not assert that "dried skim milk is synonymous with protein." They did assert, however, and still maintain that in kwashiorkor, which may be regarded as the archetype of protein malnutrition, reconstituted dried skim milk is a remarkably effective agent in initiating cure, and achieves results which no vitamins or known accessory food factors will achieve when added to the previously deficient diet. This subject is further discussed in the next section.

The importance of protein malnutrition as an important and even basic cause of ill-health in large areas of the under-privileged world, including both the temperate and tropical zones, have been reviewed by (22, 23). In this and in the reports referred to in the section on Protein Foodstuffs consideration is given to the possible widespread ill-effects of chronic protein malnutrition in older children, adolescents and adults. As would be expected if chronic protein malnutrition were a widespread cause of ill-health almost every system of the body is affected in the tropical and under-privileged world by diseases which are seldom if ever encountered in temperate climates and more privileged communities. Moreover, the fact that many of these diseases are encountered in underprivileged parts of the world right outside the tropical belt suggests that strictly tropical diseases are not important in their etiology. Undoubtedly the majority of them are of multiple etiology. Parasitic, bacterial, and virus infections, perhaps supplemented in some cases by toxic contaminants of food, probably play a part in etiology, but

the most important underlying cause and the one which can most readily be remedied is protein malnutrition.

The mechanisms of protein malnutrition have been the subject of a conference in Jamaica, the report of which will appear shortly (26), and some aspects of the problem have been discussed by Gyorgy (17). The subject is undoubtedly complex. Some of the general questions which will require answers in the work of the next decade include the following. (*a*) What are the minimum requirements of each of the so-called essential amino acids at different ages, in human nutrition. (*b*) What are the nutrients including carbohydrates, fats, minerals, trace elements, vitamins, and incompletely identified accessory food factors that are necessary for the optimum utilisation of essential amino acids in human tissue formation. In other words if these other nutrients are supplied in optimum quantity what amino acids are still essential in the sense that they are necessary for tissue building and cannot be synthesised in the body.

Kwashiorkor

Progress in Kwashkorkor was reviewed by Brock at the Amsterdam Congress (27), and a monograph on kwashiorkor has just appeared by Trowell, Davies & Dean (19). The subject is also extensively referred to in most of the references mentioned in the section on Protein Malnutrition. The name is now very widely accepted to denote a clinical syndrome resulting from protein malnutrition in the postweaning stages of infancy. It occurs in underprivileged parts of the world both within and without the tropics where customary methods of weaning are based largely or wholly on starchy paps without reasonable additions of animal or first class vegetable protein. The name is sometimes applied also to a similar or related syndrome occurring in older children or even in adults. Its great prevalence in the age period six months to four years is probably a result of the high needs for protein in the rapidly growing phase of life and also to the difficulty of finding substitutes for cow's milk in the postweaning diet of infants in those areas of the world where cow's milk is scarce or not available. The term protein malnutrition has already been defined and discussed, and it must be emphasised again that it is used in the sense of a low ratio of calories drawn from protein foodstuffs to calories drawn from carbohydrates. Although parasitic, bacterial, and virus diseases, toxic food contaminants, and even climatic stresses may play a contributory role in etiology, the syndrome is seldom if ever encountered except in the presence of dietary protein malnutrition. In his review Brock (27) has pointed out that the pathology and biochemistry are consistent with the view that it is a basic entity although its clinical appearances may be greatly modified by complicating vitamin and calorie deficiency. The strongest argument, however, in favour of kwashiorkor being an entity resulting from dietary protein malnutrition as defined is the excellent response to skim milk or reconstituted dried skim milk. It is now clear that dried skim milk contains a factor or factors which initiate

cure in the great majority of cases provided that (*a*) complicating infections can be controlled (*b*) the child is not moribund on admission and (*c*) serious fluid and electrolyte imbalance, usually resulting from diarrhoea, can be corrected in the early stages. The term "initiation of cure" is deliberately used because it is not claimed that skim milk can restore the child to perfect health. What is claimed is that, with the reservations made above, reconstituted dried skim milk contains all the factors necessary to turn a downward and ultimately fatal course into a clinical remission. Doubtless other factors are required for the restoration of normal health and physique. These factors are usually obtained from mixed feeding as the infant's appetite recovers and its apathy disappears.

Interest should now centre on whether a similar clinical remission can be initiated by synthetic amino acids. If this proved to be the case then it would be clear that the limiting factor in the diet which leads to fatal kwashiorkor is a certain minimum quantity and pattern of amino acids. This would not, of course, mean that the other nutrients present in dried skim milk, which include carbohydrate, traces of fat, minerals, vitamins, trace elements, and unidentified accessory food factors of vitamin-like nature, do not contribute to recovery. It would mean only that the final limiting factors are amino acids. The potassium content of the dried skim milk almost certainly contributes something to rectify the dangerous state of potassium depletion which is encountered at least in those cases of kwashiorkor which have had severe diarrhoea [Hansen & Brock (20)]. Vitamin and vitamin-like accessory food factors may also economise amino acids.

Animal and Vegetable Proteins

The growing realisation of the inadequacy of world production of animal proteins and the need to investigate the extent to which protein-rich vegetables such as legumes can replace animal foodstuffs is apparent in several of the reviews referred to under the heading, Protein Foodstuffs. The special application of this need to the problems of feeding the postweaning child in areas where animal milk is not readily available is exhaustively discussed by Dean (36) and summarised in the following words:

> The trials set out to assess the value of the cereal and soya mixtures, in comparison with fresh cow's milk, by answering the three questions, whether it was possible to feed young children successfully on diets in which there was no milk or other animal protein, whether the addition of dried skimmed milk to the cereal and soya mixtures was an advantage, and whether, if the trypsin inhibitor was removed from the soya, the milk could be omitted or reduced in amount. The questions were not answered completely, but so much information was gained of a nature likely to be helpful in any future attack on the main problem that it was decided to report in full the comparative failures as well as the successes.

A useful section in the review is the modern reconsideration of the value of malting processes in rendering cereal starches suitable for consumption by the young infant. This review is timely because although dried skim milk can

readily be made available in underprivileged parts of the world for the cure of kwashiorkor, it is unlikely that the disease will be prevented except by the use of vegetable milk substitutes, especially in the tropical underprivileged world where fresh animal milk is often unobtainable. Dean has shown (37) in Uganda that the banana "starchy pap" which causes the disease can be rendered curative by the addition of soya bean flour. Presumably, therefore, soya bean and perhaps a variety of legumes may be suitable for the post-weaning diet of the infant. One interesting but incomplete piece of evidence on this score is yielded by a study of the Bushmen of the Okavango district of Southwest Africa (38). They showed no evidence of kwashiorkor among their postweaning infants, although since these primitive people have never domesticated any animal, it is certain that the infants receive no animal milk in their postweaning diet. It appears that they are fed on a "vegetable milk" prepared from the fruits of naturally grown leguminous plants. Since the Bushmen have never cultivated any crops the infants certainly do not obtain any cereal foods. It cannot be averred that the infants consume no animal protein since their parents subsist largely on the products of the chase. The mothers, however, maintain that the postweaning diet for a considerable period is entirely derived from vegetable sources. It seems highly likely that leguminous and other good vegetable proteins should be adequate for infant nutrition, but the certainty of this surmise remains undemonstrated. Presumably one vegetable protein will be better than another and presumably mixtures of vegetable proteins may be better than a single vegetable protein. Much work of vast public health importance remains to be done in this field. Analysis of the curative capacity of individual fractions of dried skim milk may provide a short cut to knowledge of the extent to which vegetable proteins may be satisfactory for the nutrition of the postweaning infant. A not unreasonable theory of human ecology might be that population pressure, in emphasising the need for calories, may have led to the cultivation of high calorie starches and consequent neglect of high protein vegetables such as the legumes in the feeding of the postweaning infant in all those regions where animal milks have not been used.

The interest in vegetable proteins as an answer to protein malnutrition in underprivileged regions and interest in the mode of action of vitamin B_{12} has led to a new interest in the health of voluntary vegetarians in privileged regions. Two papers at the Amsterdam Conference (27) on the effects of completely vegetarian diets on human subjects, one from England (F. Wokes) and one from Holland (J. F. de Wyn,) link up with two papers by Hardinge & Stare from America (39), in a study of the extent to which human beings are able to maintain good health without the consumption of animal proteins over long periods of time. The term vegetarianism is often misapplied to what is more correctly called ovo-lacto-vegetarianism where animal proteins are supplied either freely or to a limited extent through the consumption of eggs and milk. True vegetarianism (veganism) in which protein of animal origin is not consumed at all over a period of several years is

much less common and has only recently been studied in any detail. The American study found that the pure vegetarian diet could sustain normal health and development but noted that weight and physique were poor in the adolescents of the pure vegetarian group. The serum cholesterol was significantly lower for each age group in the subjects on pure vegetarian diets. In the Dutch study those who had been on strict vegetarian diets for from two to five years (vegans) were compared with lacto-vegetarians who on request consumed no animal food for several months (from two to six months). Fifteen per cent of the vegans had signs of minor degree which could be ascribed to malnutrition. Serum chemistry in general revealed no evidence of deficiency although there were some differences in serum protein pattern and the vegans had lower hemoglobin levels with some macrocytosis. Surprisingly the B_{12} level of the serum was low in only two of the 12 vegan subjects. The English study, on the other hand, found a considerable incidence of paraesthesiae and sore tongue with macrocytosis and low blood values for vitamin B_{12} .There appeared to be considerable variability from subject to subject since among 150 persons some vegans had remained healthy after five years on the diet while others had died. In discussion of the two papers at the Congress it appeared that the percentage of calories from protein was lower in the British group than in either the Dutch or the American vegans, an observation which may account for the greater incidence of recognisable abnormality in the British group. Wokes reported further that the ratio of thiocyanate nitrogen to total nitrogen in urine was raised in those subjects whose blood vitamin B_{12} was reduced. The total ketoacids in blood were measured as hydrazones before and after oral administration of glucose, and blood tyrosyl derivatives were estimated before and after oral administration of tyrosine. Raised values were found in some of the cases. It was suggested that the raised urinary thiocyanate nitrogen might be related to the theory that improvement in the biological value of vegetable proteins by addition of vitamin B_{12} may be attributable to transmethylation involving cyanolysis. Discussion at the Congress drew attention to the fact that people with the psychological motivation of vegans may well be unreliable in their evidence about the diet which they have consumed. Nevertheless, the studies are of great interest in relation to the respective roles of vegetable and animal protein in meeting the world wide prevalence of protein malnutrition.

Lipotropic Factors and Lipotropic Activity

The last year has seen an important discussion and widening of the use of the term "lipotropic." Best, Lucas & Ridout (40) state that the word was introduced by Best, Huntsman and Ridout to describe "substances which decrease the rate of deposition and accelerate the rate of removal of excess liver fat." The term is not applicable to "a change in lipid content of a fat-laden pterygium on the cornea or in the amount of depot fat in obese human subjects or in animals, nor to changes in the lipid concentration of the blood

or generally in the body," nor to "the growth effect of choline." It is further suggested that a substance which is not a lipotropic agent may exert a lipotropic effect by increasing the rate of formation of, or potentiating in some way the action of, a lipotropic agent. Best *et al.* are in difficulties in cataloging lipotropic agents. While accepting "that choline, betaine, methionine and, under some conditions vitamin B_{12} and inositol protect rats in varying degrees against the development of fatty livers," they point out that "interference with the function of hepatic cells by means other than deficiency of lipotropes may lead to fatty livers." This statement is exemplified by the fatty liver of the guinea pig on a scorbutic diet which is held not to be a result of lack of dietary choline. They hold also that although antibiotics, such as chlortetracycline (aureomycin) may modify the requirement for choline and methionine, they do not qualify as lipotropic agents because their action may still reasonably be attributed to a modifying effect on the absorption of choline or methionine. The same would presumably apply to the action of hog-mucin [Gyorgy (17)]. They state further that "the crucial experiment to determine the lipotropic activity of choline in man has not yet been performed" because "to supply the essential amino acids without choline precursors is a problem of the highest order." These points are all very relevant to a discussion below of nutritional cirrhosis in man. The widening of the use of the term which was foreshadowed by Best *et al.* has now been formulated by Wilgram, Hartroft & Best (28) in a discussion of "Dietary choline and the maintenance of the cardiovascular system in rats." The latter authors propose "that the definition of the term 'lipotropic' be broadened to include the prevention of abnormal deposits of stainable fat in all organs of choline-deficient animals in which it has been demonstrated that this result is the direct outcome of lack of choline and its precursors." This widening is very relevant to discussions of "nutritional hearts" and "diet and atherosclerosis."

The widened definition when reapplied to fatty infiltration of the liver attempts in the words, "direct outcome of lack of choline and its precursors," to clarify in theory the dilemma that a substance which is not a lipotropic agent may exert a lipotropic effect. In practice the dilemma remains.

Siderosis and Iron Intake

Clarification and redefinition of terminology is overdue in respect of such terms as siderosis, hemosiderosis, hemochromatosis, and cytosiderosis.

It is suggested that the term siderosis (sidéros; Greek = iron) should be used in a broad way to cover excess iron in a tissue or in the body generally, whether the iron present be demonstrable or not by staining in histological techniques, and whether it be intracellular or extracellular. In other words although siderosis is often demonstrable histologically the ultimate criterion is chemical analysis and involves the establishment of biochemical norms for healthy people.

The term hemosiderosis should obviously be used where it is believed that the siderosis has resulted from abnormal destruction of hemoglobin. Cyto-

siderosis is a term used particularly by Gillman & Gillman (2) to describe siderosis involving solely or particularly parenchymal cells. In discussing cytosiderosis of the liver Gillman & Gillman (2) say

> In the early stages of the disease especially as it affected the liver it was evident from our studies that large quantities of iron could accumulate in the hepatic cells while the Kupffer cells remained singularly unaffected. Such a distribution was strong presumptive evidence that either the iron was circulating in a form which could not be taken up by the cells of the reticulo-endothelial system but which could be concentrated by the liver cells, or that the iron was arising within the epithelial cells themselves. This latter possibility was more acceptable for the several reasons mentioned previously. Moreover this view was strengthened when evidence became available that considerable amounts of iron and copper were present in the mitochondria and microsomes (Claude, 1944).

The above description is in the context of a complex nutritional disorder widely prevalent in the southern Bantu of Africa and associated with pellagrous dermatosis. The use of the term hemochromatosis obviously implies, in the context of disorders of iron metabolism, excessive stainable iron in the tissues derived from abnormal breakdown of hemoglobin or failure of excretion of iron derived from normal hemolysis. Sheldon applies the term to what he believes to be an inborn error of iron metabolism, but Strachan and subsequently the Gillmans (2) used the term extensively for the hepatic siderosis which is so prevalent in the southern Bantu of Africa. The need for clarification of terminology is pointed out by a recent paper by Higginson *et al.* (41). They traced the history of knowledge and views on the extensive deposition of hemosiderin in the viscera of the South African Bantu and produced further evidence for the nonidentity of this condition with idiopathic hemochromatosis, the hemosiderosis of certain anemias, and that which follows multiple blood transfusions. They have adopted the view first formulated by Walker & Arvidsson (42), that the main reason for the siderosis is dietary iron overload. Amongst the South African Bantu the intake of iron is surprisingly high, as much as 200 mg./diem, mainly as the result of leaching of iron from the utensils used during the preparation of meals and fermented cereal products. They suggest that the iron storage of Bantu siderosis occurs principally in the reticulo-endothelial system and liver and that it is not primarily a parenchymal siderosis as appears to be the case in classical hemochromatosis. These observations and views appear at first glance to be in conflict with the cytosiderosis concept of Gillman & Gillman in which it is postulated that the hepatic siderosis of the South African Bantu is attributable to disordered metabolism of the hepatic cell. The conflict may not be as real as at first appears since the absorption and perhaps the metabolism of the excess dietary iron may be determined by the coexisting malnutrition. Thus, for example, in animals, Gubler *et al.* (43) have suggested that pyridoxine deficiency may lead to excessive absorption of iron, while Finch *et al.* (44) have suggested that a low ratio of phosphorus to calcium in the diet may encourage iron absorption. In relation to the latter observation it is interest-

ing to recall that the ratio of iron to phosphorus in the intestine is of importance both in animals and in man. A high ratio can produce rickets in rats [Brock & Diamond (45)] and impaired phosphorus absorption in man [Brock (46)]. On the metabolic side there is the problem of the relationship between "nutritional cirrhosis" and "siderotic cirrhosis." Discussion of this point must be against the background observation that "nutritional cirrhosis" and its apparent sequel, primary carcinoma of the liver, is prevalent throughout a large part of the African continent whereas siderosis is patchy in its distribution. This suggests that the cirrhosis may be attributable to locally varying patterns of nutritional disorder such as protein malnutrition, perhaps combined with toxic factors, whereas the siderosis may be a result of dietary iron overload.

Another possible cause for the patchy distribution of "siderotic cirrhosis" may be a variable balance between blood loss, hemolysis, and hemopoiesis resulting from tropical parasitic diseases. Among the South African Bantu who show siderotic cirrhosis, the prevalance of tropical parasites causing hemolysis and blood loss is low, while hemoglobin, serum iron, and total iron-binding capacity and iron intakes are high (42). In Uganda on the other hand "nutritional cirrhosis" is prevalent without siderosis (30), malaria is prevalent, and dietary iron overload has not been demonstrated. In the Gold Coast Colony "nutritional cirrhosis" and "siderosis" are reported from a region where tropical parasites are endemic and high iron intakes are presumed (47). The relationships between "tropical cirrhosis" and "nutritional cirrhosis" have been discussed further by Brock (21).

Another aspect of the work of Walker & Arvidsson (42) is that it confirms earlier work (48, 49) that dietary iron overload can overcome the regulatory mechanisms for intestinal iron absorption (50, 51). This regulatory mechanism appears to operate only up to a certain threshold of dietary iron intake.

There has been no certainty in the past that siderosis in itself is harmful to the body although this has been presumed. The results of treatment of classical hemochromatosis by repeated blood letting have recently, however, been so successful (52) that there can be no serious doubt about the toxic action of iron when retained in the body in large quantities. The evidence so far applies only to idiopathic hemochromatosis, but it is reasonable to make the same assumption for the hemosiderosis which follows multiple blood transfusions. It now becomes a matter of great interest whether any of the ill-health encountered in the Southern Bantu with siderosis could be improved by repeated venesection. It would be difficult, however, to obtain an unequivocal answer in view of the complexity and chronicity of the malnutrition from which these people suffer, and the virtual certainty that the ill-health is attributable in large part to chronic protein malnutrition even without dietary iron overload.

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BIOCHEMISTRY OF THE STEROID HORMONES[1,2]

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INTRODUCTION

The biochemistry of the steroid hormones continues to engage the efforts of a large group of research workers. Attention has been directed in the past two years to the biosynthesis of the steroid homones (particularly those of the adrenal cortex), to their distribution and metabolic fate in the animal organism, and to the intimate nature of their effects on metabolism. General reviews of wide areas of steroid biochemistry which have appeared in the past two years include those of Dorfman & Ungar (1), Fukushima & Rosenfeld (2), Lieberman & Teich (3), and Roberts & Szego (4).

The present report will be devoted largely to consideration of the metabolism of the steroid hormones. Chemical and biological assay procedures will be referred to briefly, where knowledge of the methodology employed in these studies seems essential for interpretation of the data. Topics which will be omitted or only touched upon include microbiological and chemical synthesis of the steroids, their intimate effects on metabolic processes, and their gross morphological, physiological, and pharmacological effects in the organism. Clinical aspects will also be almost completely ignored. Comprehensive reviews have appeared recently on chemical synthesis (5 to 9),

[1] The survey of the literature pertaining to this review was completed in November, 1954.

[2] The following abbreviated forms have been used in this review: ACTH (adrenocorticotrophic hormone); ATP (adenosinetriphosphate); aldosterone (Δ^4-pregnene-11β,21-diol-3,20-dione-18-al); allotetrahydrocorticosterone (allopregnane-3a,11β,21-triol-20-one); androstenedione (Δ^4-androstene-3,17-dione); 11β-hydroxyandrostenedione (Δ^4-androstene-11β-ol-3,17-dione); cortisol (17-hydroxycorticosterone); cortisone (11-dehydro-17-hydroxycorticosterone); DOC (11-desoxycorticosterone); 17-hydroxy DOC (17-hydroxy-11-desoxycorticosterone); DPN (diphosphopyridine nucleotide); dihydrocortisol (pregnane-11β,17α, 21-triol-3,20-dione); dihydrocortisone (pregnane-17α,21-diol-3,11,20-trione); dihydro-17-hydroxy DOC (pregnane-17α,21-diol-3,20-dione); etiocholanolone (etiocholane-3α-ol-17-one); pregnanediol (pregnane-3α,20α-diol); pregnanetriol (pregnane-3α,17α,20α-triol); pregnenolone (Δ^5-pregnene-3β-ol-20-one); TPN (triphosphopyridine nucleotide); tetrahydrocorticosterone (pregnane-3α,11β,21-triol-20-one); tetrahydrocortisol (pregnane-3α,11β,17α,21-tetrol-20-one); tetrahydrocortisone (pregnane-3α,17α,21-triol-11,20-dione); tetrahydro-17-hydroxy DOC (pregnane-3α,17α,21-triol-20-one).

[3] We wish to thank Mr. R. Lewis, Miss D. Mewshaw, and Mrs. B. Schneider of the Biomedical Library, University of California, Los Angeles, for their aid in checking the bibliography. Valuable stenographic and abstracting assistance was rendered by Mrs. E. Beaver, Mrs. P. Reich, and Miss P. Vlassopulos.

microbiological synthesis (10), chemical and physical methodology (11 to 18), bioassay (19, 20), biochemical and physiological action (3, 4, 21 to 25), and clinical aspects (24, 26, 27). A brilliant exposition of current problems in steroid biochemistry has been presented by Pincus (28).

BIOSYNTHESIS AND SECRETION

Knowledge of the mechanisms and pathways involved in biosynthesis of the steroid homones has developed rapidly with the application of microchemical procedures and isotope methodology to this problem. The preponderance of interest continues to center on factors which regulate the elaboration of adrenocorticosteroids. However, an increasing amount of attention has currently been directed to steroidogenesis in other endocrine organs.

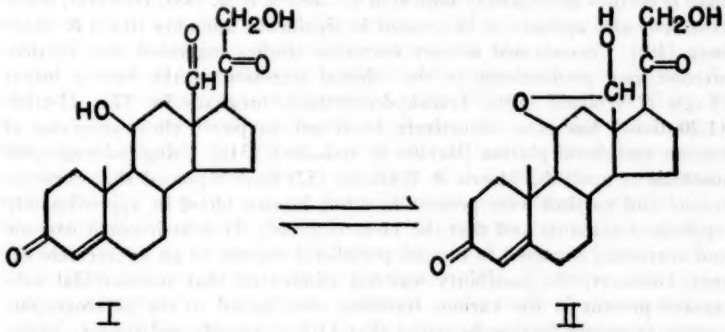
Adrenocortical Steroids

The genesis of the adrenocortical secretions has been exhaustively and capably reviewed by Hechter & Pincus (29). In addition, several other reviews of this field have appeared recently (1, 2, 3, 9, 22, 30).

The nature of adrenocortical secretion.—Approximately 30 steroids have been isolated from adrenal glands. Although many of these may appear in the secretions of the adrenal cortex at some time, it has become increasingly evident that two of these steroids account for 60 to 100 per cent of the total corticosteroid production in all species of animals studied and under widely varying conditions. These steroids are corticosterone (Compound B) and cortisol (Compound F, hydrocortisone, 17-hydroxycorticosterone). Principally as a result of the investigations of Bush, the concept has arisen that the ratio of cortisol to corticosterone secreted into the adrenal vein is normally characteristic of the species under investigation. This ratio has been reported to range from 10 to 20 in human cancer patients given ACTH[2] [Romanoff *et al.* (31)], in the rhesus monkey [Bush (32, 33)], and in sheep [Bush (32); Bush & Ferguson (34)], to be <0.05 to 0.10 in the rat and rabbit (32, 33), and to occupy the intermediate range from 1 to 10 in the bovine [Bush (32); Hechter *et al.* (35)], cat (32, 33), ferret (32, 33), and dog [Bush (32); Zaffaroni & Burton (36); Farrell & Lamus (37)]. It must be noted, however, that these ratios have been calculated from analyses made on a limited number of individuals in each case. Normal variations among members of the same species may be such as to invalidate the above generalization [cf. Hechter *et al.* (38)]. Since stress-induced release of ACTH would seem to be unavoidable during the collection of adrenal vein blood, the direct investigation of adrenal cortical function under normal, resting conditions appears to be impossible.

In addition to cortisol and corticosterone, a wide variety of other steroids has been detected by paper chromatographic techniques in adrenal vein blood and in perfusates of isolated adrenal glands. Although most of these are probably secreted in relatively minute quantities or not at all under normal circumstances, their physiological importance may be great. This con-

clusion is substantiated by the recent discovery of electrocortin in the adrenal venous effluent of the dog [Simpson *et al.* (39); Farrell & Richards (40)]. The structure of this potent mineralocorticoid has been shown by Simpson *et al.* (41) to be Δ^4-pregnene-11β, 21-diol-3,20-dione-18-al (see Formula I), which, in solution, appears to be in equilibrium with the 11-18-hemiacetal form (see Formula II). Electrocortin, presumably responsible for the potency of the so-called amorphous fraction of the adrenal cortex (42), has been given the trivial name "aldosterone" (41). Other corticosteroids whose presence has been suggested on paper chromatograms of adrenal vein blood include 17-hydroxy DOC (17-hydroxy-11-desoxycorticosterone, Reichstein's Substance S) in the dog (37, 40), cortisone (Compound E, 11-dehydro-17-hydro-



I II

xycorticosterone) in dog, cat, monkey, and sheep (32), 11-dehydrocorticosterone (Compound A) in the dog, rat, and cat (32, 33) and the ring A-reduced tetrahydro derivatives of cortisone and cortisol in man (31). The presence of certain of these, e.g., cortisone, has not been incontrovertibly demonstrated. Thus, aldosterone and several other corticosteroids of similar mobility appeared in the "cortisone spot" on paper chromatograms (cf. 32, 37). Though sought, cortisone could not be detected in the adrenal venous effluent in man (31). Evidence has also been presented for the occurrence of 17-ketosteroids in the adrenal venous effluent of man after ACTH administration (31), in the dog (38), rat, sheep, and cat (32, 33), and in cow adrenal perfusates containing ACTH [Bloch, Dorfman & Pincus (43)]. These have been tentatively identified as androstenedione [Δ^4-androstene-3,17-dione (31, 43)], adrenosterone (38, 43), and 11β-hydroxyandrostenedione [Δ^4-androstene-11β-ol-3,17-dione (31, 32, 33, 38, 43)]. It should be pointed out that some of the trace steroids found in adrenal vein blood may be formed elsewhere in the body. Measurement of adrenal A-V differences, where possible, would seem to be essential for the accurate assessment of adrenal secretory capacity (cf. 40). However, a similar variety of corticosteroids is released on incubation of slices or homogenates of adrenal tissue obtained from

the dog [Brady (44)], cow [Haynes *et al.* (45)], hog [Haines (46)], and rat [Heard *et al.* (47)]. Under these conditions, additional steroidal compounds have been detected, and the "trace corticosteroids" were released in much greater quantities (44).

Many measurements have been made of the concentrations of the corticosteroids and their metabolites in human peripheral blood, both in health and disease. This procedure has certain obvious advantages compared to similar analyses carried out on adrenal vein blood. However, the contribution of extra-adrenal factors to the steroid composition of peripheral plasma cannot be accurately assessed. There is general agreement that the corticosteroid present in largest concentration in the peripheral plasma of man is cortisol as originally indicated by Nelson *et al.* (48). However, corticosterone also appears to be present in significant amounts [Bush & Sandberg (49)]. Unconfirmed urinary excretion studies suggested that corticosterone may predominate in the adrenal secretion of the human infant [Eagle & Wolfson (50)]. Tetrahydrocortisone(pregnane-3α, 17α, 21-triol-11,20-dione) has been tentatively identified on paper chromatograms of human peripheral plasma [Bayliss & Steinbeck (51)]. Using polarographic methods of analysis, Morris & Williams (52) have reported that corticosterone and cortisol were present in adult human blood in approximately equivalent amounts and that the 11-ketosteroids, 11-dehydrocorticosterone and cortisone, occurred in human peripheral plasma to an appreciable extent. However, the possibility was not eliminated that nonsteroidal substances present in the various fractions contributed to the polarographic values. It should further be noted that 11-ketosteroids and the tetrahydro derivatives of cortisone and cortisol may represent extra-adrenal metabolites of adrenal secretory products (cf., however, 31). In addition to the corticosteroids, 17-ketosteroids, at least partially of adrenal origin, have been reported in peripheral plasma of man [Gardner (53, 54); Migeon & Plager (55, 56)].

The control of adrenocortical secretion.—Physiological and pathological variations in the rate of adrenocortical secretion have been estimated by analysis of urinary metabolites, changes in peripheral blood levels of corticosteroids, renal arterio-venous differences, corticosteroid levels in the adrenal venous effluent, and corticosteroid release by adrenal glands or tissue *in vitro*. In certain of these procedures, it would appear that only the maximal output of the adrenal gland can be estimated, since the manipulations involved result in the release of pituitary ACTH. As might be anticipated, levels of "normal" corticosteroid production reported by different investigators, even when using similar techniques, cover a wide range.

On the basis of urinary analyses and of a number of assumptions (*vide infra*), Dorfman (57) has calculated the probable daily production of neutral steroid hormones of adrenal and gonadal origin in the human. Corticosteroid production was estimated at approximately 20 mg. per day in both sexes. In addition, the production of C_{19} compounds of adrenal origin (e.g., adreno-

sterone) was calculated to be about 7 mg. per day. The rate of secretion of androstenedione (supposedly of both adrenal and testicular origin) was similar to that of the corticosteroids (cortisone and cortisol). In aged men and women, all values were markedly reduced. It should be noted, however, that the data of other investigators revealed little or no influence of age on urinary metabolites of glucocorticoids (58, 59). In addition, Nelson[4] has found no decrease in circulating 17-hydroxycorticosteroids in aged men. Simultaneous measurements of 17-hydroxycorticosteroids in renal vein and peripheral arterial blood samples from normal human subjects permitted an estimate of adrenal secretory rate equivalent to 15 to 25 mg. of cortisol per day [Bondy & Altrock (60)]. The normal level of aldosterone secretion in man was low, judging from peripheral blood levels [Simpson & Tait (61)] and from urinary excretory data [Axelrad *et al.* (62)]. Deprivation of sodium appeared to increase the output of this hormone (62, 63).

Calculations of adrenal secretory rate based on the analyses by Romanoff *et al.* (31) of corticosteroids in adrenal vein blood of man were 5 to 10 times greater than the above values (cf. 29). Similarly, relatively high values have been calculated by Bush (33, 34) for the rate of corticosteroid secretion in a number of species. Thus, about 1 to 10 mg. of corticosteroids per kg. body weight per day were secreted by the adrenals of most animals studied [cf., also, Hume & Nelson (64); Bibile & Vogt (65)]. In the rat, the secretion of corticosteroids was approximately twice the above value and was attributable principally or entirely to corticosterone (33). Measurements made in other laboratories of the adrenal secretory rate in the dog have sometimes yielded much lower values (cf. 36, 38, 66). The rate of corticosteroid release by adrenal tissue *in vitro* is comparatively low (29, 44, 67, 68, 69). Under certain conditions of perfusion, however, the beef adrenal gland may elaborate quantities of corticosteroids which approach *in vivo* levels (cf. 29).

A large number of determinations has been carried out on the levels of circulating corticosteroids in man in health and disease. Normal values for free 17-hydroxycorticosteroids in peripheral plasma of adult or aged men or women average about 5 to 20 μg. per 100 ml. (51, 52, 60, 70 to 81). The existence of a diurnal rhythm in the secretory activity of the human adrenal cortex, earlier indicated from urinary excretion studies [cf. Pincus (82)], has been recently substantiated by studies of circulating 17-hydroxycorticosteroids [Bliss *et al.* (73); Doe *et al.* (83); Tyler *et al.* (84)]. Klein and co-workers (72, 85) have reported that circulating 17-hydroxycorticosteroids were absent or very low in the blood of newborn infants (½ to 5 days old), but rapidly attained normal levels (within 1 to 3 weeks of birth). The minimal levels of adrenal cortical steroids in the plasma of newborn infants may in part be a reflection of depressed pituitary function in the foetus, as a consequence of exposure to elevated blood levels of adrenal steroids of maternal origin during pregnancy (52, 79). It should be noted,

[4] Nelson, D. H. (Personal Communication)

however, that Gardner *et al.* (86) reported that 17-ketosteroids were depressed in the plasma of pregnant women at term, but were normal in the foetal (cord) plasma.

In man, a reduction in plasma 17-hydroxycorticosteroids was observed in Addison's disease (60), in congenital adrenal hyperplasia (71, 87), and following adrenalectomy (88, 89). In contrast, elevated levels of blood corticoids have been noted in Cushing's disease (66, 71). Reduced levels of 17-hydroxycorticosteroids have been found in peripheral plasma (90, 91) and in the adrenal venous effluent (32, 90, 92, 93) of the adrenalectomized dog (90) and guinea-pig (91), and in the hypophysectomized dog (90, 92, 93, 94), guinea-pig (91), rat (92), and rabbit (32). The effect of hypophysectomy on the secretion of aldosterone has not been established (cf., however, 94, 95).

The injection of pituitary ACTH (49, 52, 60, 74, 75, 91, 94, 96) or the application of a variety of stresses (72, 76, 78, 79, 80, 97) resulted in elevation of circulating levels of corticosteroids in man and experimental animals. The nature of the response to ACTH varied with the dose and method of administration (52, 74, 96) and with the activity of the subject (52). Increased levels of 17-ketosteroids in peripheral plasma have also been observed after the administration of ACTH [Migeon (98)]. The role of epinephrine as a potential activator of pituitary-adrenal cortical secretion has been reinvestigated [Ely *et al.* (75); Sandberg *et al.* (77); Bacchus (99)]. Moderate doses of epinephrine in normal children (75) and adults (77), or in rats (99), produced no consistent variation in plasma (75, 77, 99) or urinary (77) 17-hydroxycorticosteroids (75, 77, 99) or 17-ketosteroids (77). Sandberg *et al.* (77) suggested that epinephrine may potentiate certain of the peripheral actions of the corticosteroids but does not normally activate pituitary-adrenal cortical secretion. This conclusion does not appear to be warranted from such meager observations in view of the accumulated evidence to the contrary.

Attempts have been made to estimate quantitatively the influence of ACTH on adrenal secretion by measurements of corticosteroids in the adrenal vein effluent of experimental animals. Bush failed to find a consistent rise in adrenal steroid secretion after the administration of ACTH to the normal rat and rabbit but observed an increase in the normal cat, dog, and sheep and in the hypophysectomized rabbit (32). Other investigators have reported a marked and rapid increase in 17-hydroxycorticosteroids in the adrenal venous blood of the normal (36, 64) and hypophysectomized (64, 94) dog after ACTH administration. In none of these instances was the normal ratio of cortisol to corticosterone significantly altered as a consequence of ACTH treatment [cf., however, Bush & Sandberg (49); Kass *et al.* (100)]. ACTH appeared to have no acute effect on the adrenal output of aldosterone (61, 62, 94, 95, 101). Stimulation by ACTH of corticosteroid production by surviving adrenal tissue appears to be similar in magnitude to ACTH effects observed *in vivo* (29, 45, 65, 67, 68, 69, 102 to 105). Proposed mechanisms for the action of ACTH on adrenal tissue will be discussed below.

Biosynthesis of the adrenal cortical steroids.—Significant progress has been made in elucidation of the pathways of hormone synthesis in the adrenal cortex (cf. 29). These studies have entailed systematic search for precursors of corticosteroids elaborated by the perfused adrenal gland and by slices and homogenates of adrenal tissue. The cell-free preparation has, in addition, permitted investigation of the character and requirements of enzyme systems involved in steroid transformations.

Hechter & Pincus (29) have proposed the following sequence for the biosynthesis of the corticosteroids in the adrenal cortex (see Fig. 1). Both C^{14}-acetate (46, 47, 106, 107) and C^{14}-cholesterol (106, 107) are convertible to cortisol and corticosterone *in vitro*. A dual pathway appears to exist for the formation of the corticosteroids from acetate, one involving conversion to cholesterol, and the other circumventing this step. Thus, the specific activity of the corticosteroids formed from C^{14}-acetate by the perfused cow

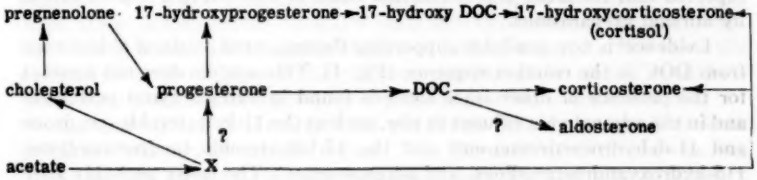


FIG. 1. Proposed biosynthetic pathway for adrenocorticosteroids (29).

adrenal was appreciably greater than that of cholesterol in the gland or in the blood perfusate [Hechter *et al.* (106)]. Moreover, Stone & Hechter (108) demonstrated that ACTH increased corticosteroid production from C^{14}-cholesterol about 20-fold, but only doubled or tripled the utilization of C^{14}-acetate for this purpose. These results suggest that some common intermediate may exist in the conversion of acetate to cholesterol and the corticoids. The nature of this steroid precursor (X) is unknown, but acetoacetate and squalene have been suggested [cf. Bloch (109)]. Hofmann & Davison (69) found that acetoacetate (and glycerol), but not acetate or isovalerate, enhanced corticoid output by rat adrenal tissue *in vitro*.

The transformation of cholesterol to the adrenal cortical steroids appears to proceed via pregnenolone [Saba *et al.* (110)]. This seems to involve scission of the side-chain prior to desaturation of ring A, since Δ^4-cholestenone was not converted to corticosterone or cortisol by the perfused bovine adrenal gland [Stone & Hechter (111)]. Pregnenolone (Δ^5-pregnene-3β-ol-20-one) was shown to be transformed to progesterone *in vitro* [Hechter *et al.* (35); Samuels (112)]. Both progesterone (35, 47, 113, 114) and pregnenolone (35) were found to give rise to corticosterone and cortisol. The hydroxylation of progesterone with the eventual formation of the corticosteroids appears to proceed in a definite sequence. Although progesterone could be hydroxylated *in vitro*, first either in the 11-position (35, 115), in the 17-position (35), or in

the 21-position [Plager & Samuels (113); Dorfman *et al.* (114)], subsequent hydroxylation was definitely limited or directed by the initial transformation. Introduction of an hydroxyl group at C-11 appears to be independent of previous hydroxylations. This is supported by the observations that 17-hydroxyprogesterone (35, 114) and 17-hydroxy DOC[2] (35, 113, 114, 116) could be converted to cortisol, and that DOC (11-desoxycorticosterone) could serve as a precursor for corticosterone (35, 114, 116). When 11-hydroxylation had taken place, however, further hydroxylation seemed to be impossible [cf. Jeanloz & Levy (117)]. The 17-hydroxyl group, once introduced, could not be eliminated (35, 113, 114, 116). Hechter & Pincus (29) have suggested that introduction of an hydroxyl group at C-17 cannot occur following that at C-21 [cf., also, Plager & Samuels (113)], on the basis of the observations that neither corticosterone (35) nor DOC (113) was 17-hydroxylated *in vitro*. However, Heard *et al.* (47) and Dorfman and co-workers (114) reported that the latter transformation could be effected to a limited extent by adrenal preparations.

Evidence[5] is now available supporting the suggested origin of aldosterone from DOC in the reaction sequence (Fig. 1). This scheme does not account for the presence of other trace steroids found in adrenal gland perfusates and in the adrenal vein effluent *in vivo*, such as the 11-ketosteroids (cortisone and 11-dehydrocorticosterone) and the 17-ketosteroids (androstenedione, 11β-hydroxyandrostenedione, and adrenosterone). The latter probably arise from 17-hydroxylated adrenal intermediates.

A number of additional steroid transformations in adrenal tissue *in vitro* has been reported (cf. 1, 29). Many of these involve hydroxylations or ring A-oxidations similar to those outlined above. Other transformations of unknown significance have also been observed, such as the 6β-hydroxylation of DOC (46), the conversion of C_{19} to C_{21} compounds, viz., dehydroepiandrosterone to cortisol [Hechter (118)], the formation of allopregnane-3β, 17α, 21-triol-11,20-dione, and allopregnane-17α,21-diol-3, 11, 20-trione from cortisone [Meyer (119)], and the reduction of pregnane-3, 20-dione [Ralls *et al.* (120)].

Enzymes active in steroid transformations in the adrenal cortex.—The wide variety of enzymatic transformations observed in the presence of adrenal tissue or homogenates *in vitro* has been tabulated by Dorfman (1). Most attention has been allotted the steroid 11β-hydroxylase, originally detected by Hayano, Dorfman & Prins (121) in slices and homogenates of beef adrenal tissue. Sweat (122) later demonstrated that this enzymic activity was localized in the mitochondrial fraction of adrenal homogenates [cf., also, Hayano *et al.* (123)]. On the basis of experiments carried out with adrenal homogenates (123, 124) and acetone powders prepared from adrenal mitochondria (125), Hayano & Dorfman concluded that the active enzyme was a TPN[3]-linked dehydrogenase, which required fumarate as a specific hydro-

[5] Wettstein, A., and Anner, G., *Experientia*, 10, 397–416 (1954).

gen acceptor for the reduced coenzyme and was inactive in the absence of oxygen. However, other intermediates in the Krebs cycle can substitute for fumarate in whole adrenal homogenates [Kahnt & Wettstein (126)] and in washed preparations of ox adrenal mitochondria [Brownie & Grant (127)]. The latter workers also demonstrated that dinitrophenol inhibited enzyme activity without affecting oxygen consumption. On the basis of these and other observations, Brownie & Grant (127, 128) suggested that operation of the Krebs cycle normally sparked steroid 11β-hydroxylation by providing high energy phosphate bonds and that fumarate might be an extraordinary requirement for hydroxylation in damaged mitochondrial preparations where the normal electron transporting mechanisms were disorganized.

The stereochemical mechanism involved in the 11β-hydroxylation of DOC is unknown. It apparently does not involve stepwise dehydrogenation at positions 9:11 followed by the asymmetric addition of water, as originally suggested by Hayano & Dorfman (124). Thus, these investigators (125) have since demonstrated that $\Delta^{9(11)}$-anhydrocorticosterone and $\Delta^{9(11)}$-anhydrocortisol were not hydrated in adrenal homogenates. Furthermore, the incubation of 17-hydroxy DOC with heavy water under similar conditions yielded cortisol without deuterium in a stable position in the molecule.

The 11β-hydroxylating enzyme of the adrenal giand was found to occur in the medulla as well as in the cortex (124). The activity of the enzyme was diminished by large concentrations of substrate (115, 124, 127), apparently as a result of inhibition of reactions in the citric acid cycle [Brownie, Grant & Davidson (115)]. Progesterone was a more powerful inhibitor than DOC (115). The enzyme exhibited maximal activity toward C_{21} steroids possessing the α-ketol side-chain (124). C_{19} compounds, such as testosterone (124, 129), androstenedione (124, 130), androsterone (29), epiandrosterone (131), and dehydroepiandrosterone (132) were less readily hydroxylated. Steroids possessing a 20, 21-glycol side-chain were resistant to 11β-hydroxylation (124). The 17- and 21-hydroxylating systems of the adrenal cortex have been studied by Plager & Samuels (112, 113). These enzymic activities did not appear to be associated with particulate cellular constituents (cf., also, 114). Both enzyme systems required TPN (or ATP plus DPN[2]) for optimal activity.

Samuels and co-workers (112) have also studied a potent ring A-oxidizing system found in the adrenal cortex as well as in other steroidogenic tissues, such as testis, corpus luteum, and placenta. This enzyme system was shown to convert certain Δ^5-3β-hydroxy steroids to the corresponding Δ^4-3-ketones, e.g., pregnenolone to progesterone (112) and dehydroepiandrosterone to androstenedione (112, 132). Enzymic activity was present in soluble supernates of adrenal homogenates, was dependent upon the presence of DPN as a hydrogen acceptor, and was enhanced by ATP. Huseby, Samuels, & Helmreich (133) observed that the steroid 3β-hydroxy dehydrogenase activity of adrenal tumor transplants in mice was roughly proportional to the androgenic character of the transplants.

The capacity of adrenocortical tissue to convert cholesterol to pregnen-

olone was found by Saba, Hechter, & Stone (110) to be associated with the mitochondrial fraction.

Control of corticosteroid biosynthesis.—The sites and mechanisms of action of ACTH in corticosteroidogenesis are unknown. Stone & Hechter (108) demonstrated that ACTH markedly stimulated the conversion of C^{14}-cholesterol to corticosteroids in the perfused bovine adrenal, but had relatively little effect on the transformation of C^{14}-acetate or C^{14}-progesterone to the same end-products, or on the conversion of acetate to cholesterol. It was suggested that ACTH may activate an early step between cholesterol and progesterone and that, under basal conditions, the normal pathway for corticosteroid production from acetate may circumvent cholesterol [cf. also, Ungar & Dorfman (134)]. Consistent with the former hypothesis were the observations of Lyons *et al.* (135) and Zarrow & Lazo-Wasem (136) that administration of ACTH to gonadectomized rats resulted in evidences of progestin secretion. On the other hand, Kass *et al.* (100) proposed that chronic stimulation of the adrenal cortex may activate later transformations in the reaction sequence as well. The reversal of the ratio of corticosterone to cortisol in the adrenal vein of the rabbit under prolonged ACTH treatment was attributed to a marked increase in cortisol formation, presumably resulting from selective stimulation of the 17-hydroxylating system. Hechter & Pincus (29) suggested that normal species differences in this ratio may be related to the relative affinities of the 17- and 21-hydroxylating enzymes for progesterone in different animals. ACTH accentuates urinary excretion of pregnanetriol (pregnane-3α,17α,20α-triol), presumably derived from 17-hydroxyprogesterone, in the adrenogenital syndrome (*vide infra*), where 21-hydroxylation seems to be impaired [cf., Dorfman (137)].

The corticosteroidogenic action of ACTH on surviving adrenal tissue (44, 45, 47, 67, 68, 69, 138) has been shown to be oxygen-dependent (45, 68). The increased oxygen consumption [cf. Saffran & Bayliss (68); Tepperman (139)] appeared to be proportional to the amount of added ACTH [Reiss *et al.* (140)]. However, stimulation of respiration of surviving adrenal slices also occurred with proteins other than ACTH (140). An *in vitro* assay for ACTH has been proposed by Saffran & Bayliss (68), based upon the ability of ACTH to stimulate corticosteroid formation when added to rat adrenal glands (preincubated to increase sensitivity of response). The integrity of the adrenal cell seems to be required for this action. Thus, the response was eliminated by homogenization (68, 69, 141) and by freezing and thawing (cf. 45). Calcium ions appeared to be essential for the ACTH effect, but not for the basal production of corticosteroids by rat adrenal tissue [Birmingham *et al.* (67)]. These observations, and the finding that ACTH was necessary for the normal penetration of inorganic P^{32} into the adrenal *in vivo* (142, 143, 144), suggest that this hormone may ultimately exert its influence on corticosteroidogenesis by altering the permeability of the adrenocortical target cell (cf. 29).

The roles of ascorbate and pantothenate in adrenal cortical function

remain obscure [cf. Meiklejohn (145); Ralli & Dumm (146)]. Existing evidence indicates that these two cofactors may not be critically essential for corticosteroid synthesis or release under basal conditions. In addition, the acute response of the adrenal cortex to stress or ACTH does not appear to be dependent upon normal levels of adrenal ascorbic acid [cf. Clayton & Prunty (147)]. However, Schmidt & Staudinger (148) reported that steroidogenesis in adrenal homogenates was enhanced by added ascorbate. It has been suggested that this vitamin may normally serve to protect from oxidation adrenal enzymes dependent for their activity on functional sulfhydryl groups [cf. Davison & Hofmann (149)]. Pantothenic acid may play a more specific role in corticosteroid biosynthesis. A decreased capacity to respond to stress has been demonstrated in pantothenate-deficient animals [cf. Dumm *et al.* (150)]. Pantothenic acid, as a component of coenzyme A, is commonly regarded as essential for steroidogenesis in the adrenal cortex [Dumm *et al.* (150); Perry *et al.* (151)] and elsewhere [Bloch (109); Boyd (152)]. However, it should be noted that the derangement in adrenocortical function resulting from prolonged pantothenate deficiency may be one of the nonspecific effects of any nutritional deficiency. Thus, Butler & Morgan (153) found that pyridoxine deficiency in the rat resulted in a diminished response of adrenal cholesterol to ACTH administration.

Gonadal and Placental Steroids

Steroidogenesis has not been as extensively studied in extra-adrenal endocrine organs as in the adrenal cortex. The present status of gonadal and placental steroid biosynthesis and secretion is summarized briefly below (cf., also, 1, 2, 3, 5).

Progesterone.—Employing paper chromatographic methods for the separation of progesterone followed by measurement of ultraviolet absorption of eluates, Edgar (154, 155, 156) has estimated the concentration of this steroid in the blood and tissues of several species. The progesterone content of ovarian vein blood in the nonpregnant ewe reached a maximum level of about 1.7 μg. per ml., 9 to 11 days postestrus (155). A similar value was obtained in the ewe about 3 months after the onset of pregnancy, but this dropped nearly to zero during lactation 28 days postpartum (156). Values ranging from about 1 to 16 μg. were found in the follicular fluid of the cow, ewe, and sow near the time of ovulation (156). Somewhat smaller amounts were present in fresh corpora lutea of the sheep, and in formalin-preserved specimens from the elephant and the gonadotrophin-treated calf (156). The progesterone content of full-term human placentae has been estimated by similar chemical procedures to be approximately 1.0 to 1.5 mg. per kg. [Pearlman & Cerceo (157, 158, 159); Noall *et al.* (160)]. Haskins (161) reported that the progesterone content of human placentae dropped precipitously just prior to parturition and suggested that this decline was responsible for the onset of labor. Noall *et al.* (160) asserted on the basis of their isolation procedure that progesterone was present in the placenta in a stable

protein-bound form, but their findings were not supported by the data of Pearlman & Cerceo (158). Pearlman & Thomas (162) estimated that the blood contained in full-term human placentae had a progesterone level of 0.4 μg. per ml. Employing polarographic methods of analysis, Butt *et al.* (163) obtained similar values for the progesterone content of human umbilical vein blood.

Chemical methods have generally failed to reveal the presence of measurable amounts of progesterone in the peripheral blood of serveral species, including the pregnant woman (163, 164) or ewe (156), and the nonpregnant ewe (155, 156) or rabbit (156). However, Zander (165), using such methods, recently reported that blood from women in the second half of pregnancy contained 0.039 to 0.268 μg. of progesterone per ml. The latter values were somewhat lower than those obtained in several species of normal and pregnant animals, employing the Hooker-Forbes bioassay procedure (166). The higher values usually obtained by the bioassay method (cf. 167 to 170) may be attributable to the presence in the circulation of progestogenic material not possessing the spectrophotometric and polarographic properties of progesterone (156, 163). It should be noted, however, that Forbes (171) has recently demonstrated that the intra-uterine assay (166) appears to be highly specific for progesterone.

Available evidence indicates that progesterone may be formed in the corpus luteum and placenta by biosynthetic pathways similar to those involved in the production of this steroid by the adrenal cortex (*vide supra*). Bloch (172) early demonstrated that about 68 per cent of the pregnanediol excreted in the urine by a woman in the eighth month of pregnancy was derived from deuterium-labeled cholesterol fed by mouth. Heard *et al.* (47, 173) found that C^{14}-acetate as well as C^{14}-cholesterol gave rise in the pregnant mare to radioactive urinary metabolites of progesterone such as allopregnane-3β-ol-20-one. Pregnenolone may serve as the direct precursor of progesterone in the ovary and placenta as well as in the adrenal gland. This transformation was facilitated *in vitro* by the 3β-ol-dehydrogenase found in the corpus luteum (cf. 112) and placenta (cf. 112, 174), as well as in testis and adrenal (112). The conversion of pregnenolone to pregnanediol, the main excretory product of progesterone, has been demonstrated *in vivo* by Ungar *et al.* (175). The existence of a 6β-hydroxylating system in homogenates of cow corpus luteum was reported by Hayano *et al.* (176). The significance of this system, also present in the adrenal, is unknown.

Androgens.—Direct evidence has been obtained for the secretion of testosterone and androstenedione by testicular tissue. West *et al.* (177) isolated these two androgens, as well as 7-ketocholesterol, from spermatic vein blood of the dog. The androgenic activity of peripheral plasma normally appears to be very low. West *et al.* were unable to detect endogeneous testosterone or free 17-ketosteroids in the peripheral circulation of experimental animals (178) or man (179). Other investigators have since reported the presence of significant amounts of endogenous 17-ketosteroids (at least partially of

testicular origin) in human peripheral blood. Thus, Gardner (53) found that the level of these steroids ranged from 40 to 130 μg. in men, and from 25 to 100 μg. in women, per 100 ml. of plasma. The Florosil column employed by Nelson & Samuels (180) for the separation of 17-hydroxycorticosteroids has been adapted to fractionation of 17-ketosteroids (53, 55). Migeon & Plager (55, 56) reported that peripheral plasma normally contained about 30 to 70 μg. per 100 ml. of dehydroepiandrosterone (presumably mainly of adrenal origin). Estimation of substances resembling androsterone and etiocholanolone (etiocholane-3α-ol-17-one) on paper chromatograms gave values ranging from 5 to 25 μg. per 100 ml. The 17-ketosteroids apparently exist in plasma mainly in a conjugated form, from which they can be liberated by acid hydrolysis (53, 55).

The daily output of testicular steroids in man has been calculated by Fukushima *et al.* (181) and by Dorfman (57). On the basis of the isotope

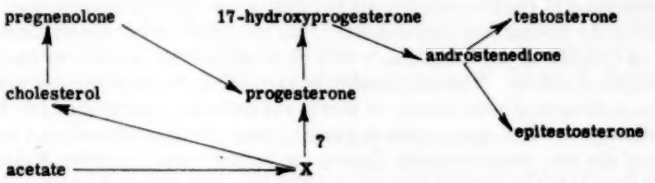


FIG. 2. Proposed biosynthetic pathway for androgens.

present in the urinary metabolites after the intravenous infusion of isotopic testosterone, the endogenous production of this steroid was estimated to be 17 to 36 mg. per day (181). Similar values were deduced from the calculations of Dorfman (57). This level appears to decline with age (cf., also 58, 59, 182).

Danby (183) early demonstrated that surviving testicular tissue from the bull was capable of producing androgenic material. Brady (184) and Savard, Dorfman & Poutasse (185) found that C^{14}-acetate was utilized for the synthesis of cholesterol (184), testosterone (184, 185), and androstenedione (185) by surviving testicular tissue. The addition *in vitro* of chorionic gonadotrophin enchanced the incorporation of radioactivity into the androgens (184, 185), but not cholesterol (184), suggesting that cholesterol may not be an obligatory intermediate in androgen synthesis. A similar situation appears to exist for the biosynthesis of the corticosteroids (*vide supra*).

The addition of dehydroepiandrosterone (183, 186) or androstenedione (183) to surviving testis tissue resulted in a significant increase in the androgenic activity of the preparation. Nissim (186) reported that pregnenolone and progesterone were inactive in this respect and suggested that dehydroepiandrosterone was converted to androstenedione through the intervention of the steroid 3β-ol dehydrogenase found by Samuels (112) in homogenates

of testis tissue. An alternative reaction sequence leading to the formation of androstenedione in the testis has been proposed by Samuels[6] on the basis of *in vitro* studies employing C^{14}-labeled steroid precursors and physico-chemical methods for the separation and identification of the products (cf. Fig. 2). In this scheme, the early steps in androgen biosynthesis appear to be identical to those proposed for cortisol formation in the adrenal cortex (cf. Fig. 1). The conversion of androstenedione to testosterone has been demonstrated in surviving testicular tissue [Wotiz *et al.* (187)].

The ring A-oxidizing system responsible for the transformation of pregnenolone to progesterone (and of dehydroepiandrosterone to androstenedione) is apparently produced in the interstitial (androgen-secreting) cells of the rat testis (112). Thus, it was present in high concentration in the cryptorchid testis after atrophy of the seminiferous epithelium had occurred. Although reduced by hypophysectomy, enzymic activity was markedly enhanced by the chronic injection of chorionic gonadotrophin, which also caused regeneration of the interstitial cells but did not restore the seminiferous epithelium. In preliminary experiments (112) the addition *in vitro* of chorionic gonadotrophin to rat testis tissue had no effect on the activity of the ring A-oxidizing enzyme. The relationship of the activity of this enzyme system to the synthesis of testosterone *in vivo* is not definitely established (cf. 133).

Estrogens.—Relatively little is known about the biosynthesis and secretion of the estrogenic steroids. Estrogenic activity was found by Rakoff & Cantarow (188) in the ovarian vein of the dog. The levels of unconjugated estrogen in human plasma have been estimated by Veldhuis (189), employing fluorometric analysis. The values obtained were probably overestimates as a result of the presence of nonestrogenic fluorogenic contaminants in the plasma extracts[7] (cf. 11). Pope & Roy (190) found unconjugated estrogenic material in extracts of cow colostrum, but not of milk. Relatively high concentrations of estriol, estrone, and estradiol-17β were found in human placental extracts by Diczfalusy (191) and Mitchell & Davies (192), employing fluorometric analysis of fractions separated by countercurrent distribution (191) or chromatography (192). A considerable proportion of the estrogens in the placenta occurred in a conjugated form, part of which (especially estriol) appeared to be protein-bound (191, 192). Estriol seems to be the predominant estrogen in women, judging from analyses of placentae (191, 192), normal (193) and pregnancy (191) blood, and normal and pregnancy urine (cf. 194).

The capacity of the perfused ovary of the sow to promote the incorporation of C^{14}-acetate into the phenolic steroids was demonstrated by Werthessen *et al.* (195). However, the specific activity of the isolated estrone and estradiol-17β was very low (3 c.p.m./mg.) compared to that of cholesterol in the perfusion medium (75 c.p.m./mg.). Heard and co-workers (47) observed that C^{14}-acetate administered to the pregnant mare gave rise to isotopically-

[6] Samuels, L. T. (Personal Communication)
[7] Goldzieher, J. W. (Personal Communication)

labeled urinary estrone, equilin, and equilenin. Estrone-16-C^{14} was not transformed, under the same conditions, to the latter to two estrogens (47). Urinary estrone isolated after intrajugular administration of cholesterol-4-C^{14} to the pregnant mare was nonradioactive (173). It would appear from these findings that the body cannot aromatize cholesterol and that the biosynthesis of the estrogens from primary precursors such as acetate must proceed via some other intermediate. Nissim (186) reported that estrogenic material was not produced upon incubation of the following steroids with minced beef ovarian tissue: pregnenolone, progesterone, dehydroepiandrosterone, or testosterone. It seems evident that the biosynthesis of the phenolic estrogens proceeds by pathways which may differ considerably from those elucidated for the nonbenzenoid steroids.

TRANSPORT

The physico-chemical and biological factors involved in the transport and distribution of the blood steroids have elicited considerable interest in recent years. A more complete knowledge of these phenomena is basic to a better understanding of steroid hormonal activity [cf. Szego & Roberts (196)].

Nature of Circulating Steroids

Considerable controversy has developed regarding the possible lipoprotein nature of at least certain of the circulating steroid hormones (cf. 4, 196). The nature of lipoprotein complexes and their biological formation were comprehensively reviewed at a conference under the auspices of the Faraday Society (197). The conclusions (cf., also, 198) may well serve to resolve some of the controversial points raised by Lieberman & Teich (3) and Bischoff *et al.* (199, 200) concerning the applicability of the term "lipoprotein" to the circulating steroid hormones.

Distribution of steroids between plasma and cells.—Scanty information is available regarding the distribution of the hormonal steroids between blood plasma and blood cells *in vivo*. The major proportion of these steroids appears to be present in the plasma (201 to 205). Contrary results may require reevaluation in view of the recent observation that the corticoids normally appear to be present exclusively in plasma, but may penetrate the red cell if blood is allowed to stand before centrifugation (204).

Free and conjugated steroids in blood.—Available evidence suggests that the steroid hormones are secreted into the blood in the free form (cf. 9, 156, 177, 188, 206) but rapidly become conjugated at least in part, presumably by passage through the liver (*vide infra*). Thus, the major corticosteroids present in human peripheral plasma appear to be cortisol and glucuronide conjugates (71) of the tetrahydro derivatives of the 17-hydroxycorticosteroids.[5] The 17-ketosteroids seem to circulate in the plasma in a conjugated form, but not as complexes with glucuronic acid (55, 56). Judged by analogy from urinary studies, they may be present principally as the sulfates (*vide infra*). A sig-

nificant portion of the blood estrogens appear to be conjugated (191, 203, 207, 208), possibly as the glucuronoside (cf. 196).

Protein-bound steroids in blood.—The earliest evidence for the existence of plasma protein-estrogen complexes in blood seems to have been presented by Brunelli (209) and by Haussler (210). Mühlbock (203), who has erroneously been quoted as obtaining similar results (cf. 3,200), failed to corroborate the extensive studies of Haussler. More recently, additional evidence has accumulated for the presence of protein-bound estrogen in the blood of man and other species (191, 193, 196, 207, 208). The probable existence of other natural hormonal steroid-plasma protein complexes is suggested by the work of Hooker & Forbes (201) on progestin, Lewis & Savard (211) on corticoids, and Gardner (212) on 17-ketosteroids.

The accumulated evidence for the existence of estroprotein (193) in human plasma may be summarized as follows. After precipitation of the plasma proteins by "salting out" procedures (207, 208), or with ethanol (191, 208), butanol (191), or acetone (208, 210), a considerable and rather constant (208, 209) proportion of the blood estrogen remained in the precipitate (cf., however, 200). This estrogen could not be liberated from the proteins by simple extraction with organic solvents, but only after the precipitate had been subjected to such drastic treatment as acid (207) or alkali (191, 208) digestion, or prolonged Soxhlet extraction with ethanol followed by ether (208). Bischoff *et al.* (200) have misquoted the findings of Szego & Roberts (208) in stating that "all the estrogen is removed from the protein upon ethanol precipitation." Actually, only one-third of the blood estrogen was removed by this procedure (208). The major portion of the estrogen present in pooled human plasma was found in the β_1-lipoprotein [Fraction III-0 of Cohn *et al.* (213)], from which it could not readily be extracted by ether (193). Lieberman & Teich (3) in describing these observations have erroneously stated that "some of the estrogen could be extracted from the protein by cold ether." Bischoff *et al.* (200) were unable to confirm the presence of estrogenic activity in the human blood plasma Fraction III-0 (193). They reported that dried protein fractions of human sera were employed in their studies. The Harvard group (cf. 214) has repeatedly emphasized the instability of the β_1-lipoprotein preparation and the ease with which the lipides present auto-oxidize and split off from the protein in the dry state or even with standing in an aqueous concentrated form in the cold. Roberts & Szego (193) originally noted that the estrogenic activity of Fraction III-0 "diminishes with time even when stored in the cold." It is perhaps pertinent to note here that although Gardner (212) found appreciable amounts of 17-ketosteroids in purified plasma protein fractions, prepared according to the scheme of Cohn *et al.* (213) but stored for long intervals, the values observed were far lower than those obtained on whole fresh plasma (53)

Doubt has been cast (3,200) on the existence of estroprotein as most of the estrogen present in blood has been shown to dialyze readily through a collodion membrane (193, 208). Lieberman & Teich (3) have incorrectly

stated that Rakoff *et al.* (207) "reported that the bound estrogens could not be separated from the protein by dialysis." In reality, the latter investigators observed that estrogens could not be ultrafiltered from serum, a finding confirmed by Roberts & Szego (193) which supports the concept of protein-binding. The argument that dialyzability of plasma estrogen is evidence against protein-binding would not appear to be justified from studies of other complex proteins. Thus, considerable amounts of other lipides dialyze out of purified lipoprotein preparations, which are nonetheless considered to be bound to the protein (cf. 197). In addition, the protein-bound nature of coenzymes has never been questioned on the basis of the ease of removal from the apoenzyme by dialysis.

The significance of the terms "lipoprotein" and "protein-binding" has also been the basis for confusion and disagreement regarding the existence of hormonal steroid-protein complexes in plasma. Bischoff *et al.* (200) maintained that the linkage of steroid to protein must be atomic in nature or via strong secondary valences to satisfy the definition of protein-binding. However, many natural blood lipoproteins, including those containing cholesterol or cholesterol esters, do not appear to be conjugated proteins and are believed to be composed of a complex mixture of loosely associated micelles (cf., 197, 198). The important criteria which may be applied to the natural lipoproteins include a relatively constant composition under natural conditions, water solubility, chemical and physical properties different from the individual components, and the intervention of metabolic processes in their formation (*vide infra*). The estrogenic steroids in blood appear to satisfy these requirements for "protein-binding."

Bischoff *et al.* and Samuels and co-workers have conducted an extensive series of experiments on the capacity of serum and of serum proteins to "solubilize" the steroid hormones. *In vitro*, albumin appears to be more effective than the globulins in dispersing (215) or binding (216) steroids. *In vivo*, this situation may be reversed, since β-globulin seems to be the most lipide-avid protein, albumin taking over this function only when the globulin is saturated [cf. McFarlane (217)]. On the basis of the distribution coefficients found for the various steroid hormones between bovine albumin solutions and peanut oil (200) and other biphasic systems (199), Bischoff *et al.* concluded that the dispersing ability of albumin for these hormones was governed strictly by solubility laws and did not involve complex formation. This conclusion would appear to be out of harmony with earlier data from the same laboratory. Thus, Bischoff & Katherman (215) reported that testosterone, and to a lesser extent progesterone, blocked the dispersion of estradiol in albumin solution. The authors state that these results were probably attributable to "competition between the secondary valence bonds of testosterone and estradiol for those of albumin." This statement would seem to imply a recognition of steroid-protein binding forces. The competition between steroid hormones for specific lipoprotein sites *in vivo* has been advanced as a possible mechanism for steroid hormone antagonisms (4, 196).

Eik-Nes *et al.* (216) reported that the capacity of different steroid hormones to bind to bovine albumin *in vitro* in general decreased with increasing polarity of the steroid. However, estradiol was an exception to this rule, being more readily bound than most other steroids tested. These observations may explain the *in vivo* finding that the acidic estrogenic hormones are physiologically active in much smaller concentrations than the other steroids and are capable of antagonizing the biological effects of large amounts of these other steroids (4, 196). The existence of steroid-protein binding forces is further substantiated by the observation that the steroid-dispersing properties of albumin *in vitro* may be altered by blockade of end groups in the protein molecule with ionic substances [Schellman *et al.* (218)]. The physicochemical properties of the steroids which may be related to their ability to bind proteins in nonbiological systems have been discussed by Turner (219).

The *in vitro* studies by Bischoff *et al.* and Samuels and co-workers have been useful in the development of steroid solutions which can be used for intravenous administration of these hormones (cf. 178, 179). Their usefulness in explaining steroid-protein interactions *in vivo* is limited by the probability that the globulins serve as the natural transporting agents for the steroid hormones at least in the human, and that metabolic intervention appears to be essential for lipoprotein associations *in vivo*.

Biological mechanisms of steroid-protein association.—The formation and transformation of the lipoproteins *in vivo* appear to be dependent upon enzymatic mechanisms. McFarlane (217) reported that cholesterol and cholesterol oleate could not be dispersed in serum *in vitro* in appreciable quantities. Other workers have also observed that cholesterol was bound (216) or dispersed (215) very poorly by plasma proteins *in vitro*. The presence of the major proportion of cholesterol in serum as lipoproteins suggests that the association between lipide and protein in the organism may be dependent upon metabolic processes. Anfinsen and co-workers (220) described enzymatic mechanisms which appear to be involved in the transformation of lipoproteins.

Evidence for the intervention of the liver in estrogen-protein binding has been summarized (4, 196). Szego (221) recently demonstrated that the incubation of estrone 16-C^{14} with rat liver mince in an homologous serum medium resulted in the appearance of radioactivity in the serum and liver proteins. This reaction was augmented in regenerating liver tissue (221) and curtailed in hepatoma (222). Protein-binding did not occur in serum alone, nor in the presence of spleen, kidney, or uterine tissue (221). The bulk of the radioactivity bound to the serum proteins in the presence of hepatic tissue appeared to be a glucuronoside complex (223), from which biologically-active estrogen was released by hydrolysis (221). Circulating protein-bound estrogen also seems to be conjugated with glucuronic acid (4,196). The protein-bound radioactivity derived from C^{14}-estrone incubated in serum in the presence of surviving rat liver tissue was present principally in the albumin fraction of the serum proteins separated by filter paper electrophoresis [Szego

& Roberts (224)]. Whether or not estrogen is naturally present in rat serum bound to albumin is unknown. It should be noted, however, that β-lipoproteins are peculiar to human plasma (197). Other serum proteins assume the lipide-transporting role of this group of proteins in animals other than man (197).

Riegel & Mueller (225) confirmed the observation (221) that rat liver preparations were capable of effecting the binding of added estrogens or their metabolites to the liver proteins. Rat kidney, uterus, and submaxillary gland preparations (225) and human placental tissue (cf. 191) were inactive in this regard. The maximal binding of metabolites of C^{14}-labeled estradiol has been observed in homogenates of rat liver fortified with TPN and an oxidizable substrate, and incubated under oxygen (225). When DPN was added as well, estradiol appeared to be rapidly oxidized to estrone before binding occurred. Under these circumstances, the protein-bound material was devoid of biological activity after release by hydrolysis (225). It should be noted that estrogen metabolites associated with the liver proteins appear to be conjugated with substances other than glucuronic acid (223, 225). The binding of estrogens to the serum and liver proteins may represent two distinct phenomena, the first related to the transport and activity of this class of steroids (cf. 4,196), and the second associated with their metabolic inactivation (cf. 225).

Distribution

Studies of the distribution and fate of exogenously administered steroids have been greatly facilitated by the use of isotopic tracers [cf. Twombly (226)]. These observations bear less on the sites of action of the hormonal steroids than on their detoxication and elimination from the body. Thus, it appears to be generally true that the steroid hormones after exogenous administration disappear rapidly from the tissues and show no tendency to localize in the target organs. Nonetheless, the investigations correlating route and manner of administration with pathways and rates of excretion may be expected to contribute to our understanding of the biological action of this class of hormones.

Progesterone and corticosteroids.—The C_{21} steroids, in common with the C_{18} and C_{19} compounds (*vide infra*), disappear rapidly from the circulation after administration. This phenomenon has been demonstrated after the intravenous injection of progesterone into man (227) or rabbit (164, 228), and in the rat (163), even after evisceration (229) or impairment of the hepatic circulation (163). Similarly, the rapid disappearance of 17-hydroxycorticosteroids from the circulation of the human (230, 231), dog (232), and rat (233) has been observed following intravenous infusion (230 to 233) or oral administration (230). Hepatectomy, but not splenectomy or nephrectomy, prevented this rapid disappearance in the rat (233). In hepatic disease in the human, evidence has been presented for delayed removal of 17-hydroxycorticosteroids from the blood (234). Sandberg *et al.* (235) reported

that patients with pernicious anemia maintained higher levels of these steroids for longer periods of time than normal subjects, after the oral administration of cortisol acetate or the free alcohol. Since this difference did not occur after intravenous administration, it was concluded that gastrointestinal destruction of the orally-administered steroids was diminished in the achlorhydric patients. It has been suggested (236) that the apparently reduced rate of disappearance of intravenously-infused cortisol in elderly men may be attributable to a decrease in the efficiency of distribution of the steroid throughout the extracellular fluid compartment of the body.

Sandberg *et al.* (237) have shown that 17-hydroxycorticosteroids did not normally pass the blood-brain barrier in man, but appeared in spinal fluid when meningeal inflammation was present. Hellman *et al.* (231) found no radioactivity in a 10 ml. sample of spinal fluid collected from a patient with chronic lymphatic leukemia, 8 hr. after the intravenous injection of cortisol-4-C^{14}. However, Baron & Abelson (238) reported the presence of "cortisone" and "cortisol" spots on paper chromatograms prepared from partially purified extracts of two 500 ml. samples of cerebrospinal fluid pooled from patients in a neurological unit. The amounts of the cortisone-like and cortisol-like materials present corresponded to a spinal fluid concentration of 0.1 to 0.2 μg. per 100 ml. and 0.2 to 0.4 μg. per 100 ml., respectively. Small amounts of corticosteroids appear in pleural, ascitic, and pericardial exudates in man (237), and in the aqueous humor of rabbit eyes (239).

The time of survival of the C_{21} steroids and their metabolites in the tissues appears to be very short. This has been demonstrated for progesterone in the intact female mouse and rat (240), in the castrated rabbit (228), and in the eviscerated male rat (229), and for cortisone [Bradlow *et al.* (241)] and DOC (47) in mice. The biological "half-life" of cortisol has been estimated to be 3 to 6 hr. in man (231, 242). Administered progesterone-21-C^{14} or its metabolites did not appear to be concentrated in the sexual target organs (240). A rapid, but transitory, concentration of progesterone or its metabolites in the adipose tissue of the castrated rabbit (228) and the eviscerated rat (229) has been observed after intravenous injection. Skeletal muscle may also selectively concentrate these substances, but to a much lesser extent (228). Significant amounts of radioactivity occurred in the pituitary of the rat and mouse, and in the adrenal of the mouse, 24 hr. after the intraperitoneal administration of progesterone-21-C^{14} (240). However, measurement of arteriovenous differences for 17-hydroxycorticosteroids in man after the infusion of ACTH, cortisol, or cortisone failed to reveal significant removal of the steroids by the peripheral tissues, even in severe inflammation (48, 112). Evidence was obtained in similar investigations for hepatic removal of administered corticosteroids in man (242) and dog (230). In the latter study, renal A-V differences were negligible. The selective localization in liver tissue of C_{21} steroids has been demonstrated following the injection into rodents of tritium-labeled cortisone (241) and of progesterone-21-C^{14} (240, 243). Hepatic concentration of exogenously-administered hor-

monal steroids would appear to be a reflection of the important role of this organ in the metabolism of the steroids, including their degradation and excretion, and possibly their activation (cf. 196, 225, 241). Adrenalectomy has been reported to be without significant effect on the disposal of cortisone-*t* in the mouse (241) or of cortisol-4-C^{14} in man (231). These observations, coupled with those of Nelson *et al.* (48, 112) on the failure of inflammation to modify the peripheral removal of the corticosteroids, would seem to provide evidence against the concept that utilization of these steroids is associated with "tissue demand" (cf. 112).

Species differences in the pathways of excretion of the C_{21} steroids have been demonstrated. In man the kidney was the major route of excretion for metabolites of isotopically-labeled progesterone (244), cortisone (244), and cortisol (231, 242). A minimal rate of biliary excretion was suggested by the detection of a small portion of the administered radioactivity in the feces of human subjects within two to four days following intravenous injection of cortisol-4-C^{14} (231, 242). In the rodent the biliary and fecal routes of catabolite excretion were prominent for the C_{21} steroids, progesterone-21-C^{14} (47, 240, 243, 245, 246), cortisone-*t* (241), cortisol-4-C^{14} (247), and 11-dehydrocorticosterone-21-C^{14} (248), after administration by the intraperitoneal (240, 241), subcutaneous (47), intragastric (243, 246, 247, 248), and intramuscular (241, 245, 247, 248) routes. However, after the subcutaneous injection of DOC-21-C^{14} into an estrous rabbit for three days, Heard *et al.* (47) observed that the urinary excretion of metabolites predominated over the fecal route over a seven-day period of collection. The intravenous administration of cortisone-*t* to mice resulted in a greater excretion of isotopic metabolites in the urine than in the feces, in contrast to the pattern obtained after intramuscular or intraperitoneal injection of the steroid (241). On the other hand, a consistently greater rate of fecal elimination of isotopic metabolites was observed following intramuscular, intravenous, or intragastric administration of cortisol-4-C^{14} (247). The bile served as a major route of excretion for metabolites of isotopic progesterone in the rat; the kidney assumed this function only after bile duct ligation (243, 245, 246). An enterohepatic recirculation has been demonstrated for metabolites of progesterone (243), cortisone (241), and cortisol (247).

Oxidative scission of the side chains of progesterone and DOC has been reported. Radioactivity has been demonstrated in the respiratory CO_2 (47, 240, 243, 245, 246, 249) and urinary urea (47, 246) of rodents given progesterone-21-C^{14} by a variety of routes. Administration of DOC-21-C^{14} to the mouse resulted in a greater recovery of $C^{14}O_2$ than that achieved with progesterone-21-C^{14} under similar circumstances (47). Similar experiments carried out with 11-dehydrocorticosterone-21-C^{14} (248) and cortisone-*t* (241) have failed to reveal the presence of significant amounts of radioactivity in the expired air of the rat (248) or the mouse (241). However, side-chain scission of cortisone has been repeatedly demonstrated by other criteria.

Androgens.—The rapid clearance from the blood of testosterone, ad-

ministered intravenously (164, 178, 181, 250) or intraperitoneally (249), has been demonstrated in several species of animals, including man (181, 250), rat (178, 249), rabbit (164, 178), mouse (249), and dog (178). West (178) found that 90 per cent of the administered steroid disappeared within 10 min. from the blood of intact dogs and rabbits. This rapid rate of disappearance was reduced only to 75 per cent even in an hepatectomized, nephrectomized rabbit. In spite of rapid diffusion of the steroid into the tissues, particularly adipose tissue (cf. 178, 229), testosterone had essentially disappeared from the body within 1 to 3 hr. in eviscerated (229) or intact (178) rats. Barry *et al.* (249) observed virtually no accumulation of isotope in any tissue other than the liver even as early as 1 hr. after the intraperitoneal administration of testosterone-4-C^{14} to mice.

The proportion of metabolites of the androgens excreted by different routes would appear to vary with the dose and route of administration and with the species of animal. Fukushima *et al.* (181) found that, within one to two days after the slow intravenous infusion of testosterone-*d*-4-C^{14} into man, 60 per cent of the administered isotope appeared in the urine. Negligible amounts were excreted by this route after the third day. On the other hand, West *et al.* (250), employing massive intravenous doses of testosterone, observed a secondary rise in urinary excretion of 17-ketosteroids during the five to seven day period following administration. In the isotope studies (181), fecal excretion of nonketonic material and other unidentified degradation products accounted for a small proportion of the administered steroid. In the human, therefore, the urinary route of excretion of the C_{19} steroid metabolites takes precedence over the gastrointestinal pathways. This situation seems to be reversed in the rodent.

In a succession of papers from Doisy's laboratory, the distribution and excretion of several C^{14}-labeled compounds of the androgen series have been investigated, including testosterone-4-C^{14} [Ashmore *et al.* (251)], 17α-methyl-C^{14}-Δ^5-androstene-3β, 17β-diol [Hyde *et al.* (252)], and 17α-methyl-C^{14}-testosterone [Hyde *et al.* (253, 254)]. A comparable pattern of elimination of isotopic metabolites was observed following intragastric administration of each of the steroids. Thus, after intragastric or intramuscular administration of 200 μg. of the labeled testosterone to intact male rats, two-thirds of the isotopic metabolites appeared in the feces over a four-day period (251). The remainder were excreted in the urine during this interval. Much the same excretory pattern had earlier been demonstrated in mice 24 hr. after the intraperitoneal administration of labeled testosterone (249, 255). Fecal radioactivity occurred principally in water-soluble degradation products which were not rendered ether-soluble after hydrolysis with acid or bacterial β-glucuronidase (249, 251, 255). The activity of the intestinal flora in degrading the steroid molecule was indicated by the observation that these hydrolytic procedures conferred ether-solubility on the majority of the C^{14}-labeled metabolites of testosterone found in bile collected by cannulation of the bile duct (251). Evidence pointing to the enterohepatic recirculation

of these metabolites has been presented (249, 251, 254). Lymphatic absorption of 17α-methyl-C^{14}-testosterone, administered intragastrically, was negligible (254). It is apparent that the greater biological activity of this androgen over testosterone, when both are given enterally, cannot be ascribed to ability of the former to by-pass the portal circulation (cf. 254). Ligation of the bile duct in rats led to striking increases in the amounts of radioactive metabolites of the androgens excreted by the kidneys (251, 252, 253).

In none of the experiments employing C^{14}-labeled androgens has isotope been detected in the respiratory CO_2. Negative data have been obtained after the intravenous infusion of testosterone-*d*-4-C^{14} into man (181), the intragastric (251) or parenteral (249, 251, 255) administration of testosterone-4-C^{14} to the rat (251, 255) or mouse (249), and the intragastric administration of 17α-methyl-C^{14}-Δ^5-androstenediol (252) or 17α-methyl-C^{14}-testosterone (253) to the rat.

Estrogens.—In general, earlier data obtained on the distribution and excretion of administered estrogens employing halogen-substituted steroids (cf. 226) have been substantiated by more recent studies with C^{14}-labeled compounds. The biliary and fecal pathways of excretion have been shown to predominate following the parenteral [Heard *et al.* (47); Valcourt & Thayer (256)] or intragastric (256) administration of estrone-16-C^{14} to mice (47) and rats (256). Comparable results were obtained after the intragastric administration of 17-methyl-C^{14}-estradiol to rats [Bocklage *et al.* (257)]. The biliary pathway was also demonstrated to be an important route of excretion for metabolites of C^{14}-diethylstilbestrol in the rat [Hanahan *et al.* (258)]. Ligation of the bile duct in the rodent led to a marked increase in the proportion of metabolites excreted by the kidney (256, 257). In some of these studies, partial degradation of the administered steroid was observed, with the formation of water-soluble fecal or biliary metabolites of relatively low molecular weight (cf. 47). Similarly, biliary metabolites, even after appropriate hydrolysis, possessed less estrogenic activity than that which would have been anticipated from the total isotope content of the bile (256, 257). Evidence suggestive of glucuronide conjugation of a portion of the biliary metabolites resulting from the administration of estrogen to rats has been presented for estrone-16-C^{14} (256), 17-methyl-C^{14}-estradiol (257), and methylene-C^{14}-diethylstilbestrol (258). In contrast, earlier studies by Pearlman *et al.* (259), using relatively massive doses, indicated that injected estrogen appeared principally in a free form in dog bile.

The portal blood system would appear to be of major importance in the intestinal absorption and enterohepatic circulation of the estrogens. Thus, in the investigations of Bocklage and co-workers (257), significant amounts of radioactive material could not be detected in lymph collected from the lacteals or thoracic duct 4 hr. or later subsequent to the administration by stomach tube of 200 μg. of 17-methyl-C^{14}-estradiol. Negligible lymphatic absorption of estrogen has also been reported in similar experiments employing C^{14}-diethylstilbestrol (258). The significant degree of localization of

radioactivity in the liver of the rodent following administration of C^{14}-labeled stilbestrol (258) and estrone (260) may have been related to the enterohepatic recirculation of the estrogen or to the demonstrated capacity of the liver proteins to bind metabolites of estrogen (221, 225).

The predominance of the gastrointestinal route of excretion for metabolites of administered estrogen in the rodent (47, 226, 256, 257, 258) and in the dog (cf. 259, 261) was not observed in the pregnant mare after a single massive intravenous injection of estrone-16-C^{14} (47). However, in the pseudopregnant rabbit, fecal recovery of ether-soluble radioactivity after subcutaneous administration of estrone-16-C^{14} was relatively small [Stimmel (262)].

A number of soft tissues, but not heart or spleen, were reported to contain trace amounts of radioactivity 24 hr. after the subcutaneous injection of estrone-16-C^{14} into mice (260). Isotope also appeared in bone at this time. The oral administration of tri-*p*-anisylchloroethylene to women resulted in the appearance of this synthetic estrogen in the adipose tissues (263, 264).

The capacity of the body to degrade the steroid nucleus was demonstrated by Heard *et al.* (47), who found radioactivity in pooled urinary urea and respiratory CO_2 after the subcutaneous injection of a large dose (1 mg.) of radioestrone into each of five mice. In contrast, other workers were unable to detect radioactivity in the expired air of rats receiving 200 μg. of 17-methyl-C^{14}-estradiol by stomach tube (257) or 5 μg. of C^{14}-stilbestrol orally or parenterally (258). These divergent results may have been attributable to the differences in the order of magnitude of the steroid and isotope dose levels (cf. 47) or to a lack of cleavage of the labeled positions in the synthetic estrogen (cf. 257).

METABOLIC TRANSFORMATIONS

The metabolic changes which the steroid hormones undergo subsequent to their elaboration by the steroidogenic endocrine organs may well be intimately associated with their biological functions. These changes may be divided into those which involve conjugation of the steroid molecule and those which are characterized by alterations in the steroid structure. The specific relationship between steroid hormone action and steroid metabolism is almost completely unknown.

Conjugation

The importance of conjugation mechanisms in the metabolism of the steroid hormones is established by the demonstration in blood of conjugates of the endogenous hormones (55, 56, 70, 71, 191, 193, 203, 208) and of intravenously-administered free steroids (179, 227), and by the excretion of steroid metabolites in significant amounts as conjugated materials (cf. 1, 11). Less attention has been directed to elucidation of the conjugation mechanisms per se than to the development of hydrolytic procedures designed to release maximal amounts of free steroid for ultimate analysis (cf. 11, 15).

However, the latter type of study has revealed the presence of a variety of steroid conjugates in human urine, some of which escape conventional methods of hydrolysis. Although enzymic hydrolytic procedures have added greatly to our knowledge of the nature of urinary steroid conjugates, incomplete recoveries have been noted even when enzymic treatment has been combined with acid hydrolysis (cf. 11, 15). In addition to the glucuronide and sulfate conjugates which constitute the bulk of urinary steroid complexes, some unusual forms have been reported (cf. 1, 265, 266, 267), including corticosteroid-amino acid compounds [Eades *et al.* (268)]. These considerations, together with the knowledge that certain of the steroids are labile to hot acid hydrolysis (266, 269, 270, 271), have spurred the search for differential methods of chemical hydrolysis (cf. 266, 272, 273, 274, 275). Resolution of possible artifacts arising during the application of some of these has not yet been achieved (cf. 11).

It is apparent from the foregoing that knowledge of the nature of urinary steroid conjugates in man and other species is far from complete. A major proportion of estrogenic metabolites in human urine appears to be present as the glucuronosides (276). Small amounts of estrogen sulfates have also been reported (276), but the validity of this observation has been questioned (277). Although there exists general agreement that conjugated dehydroepiandrosterone in human urine occurs almost exclusively as the sulfate (266, 278), quantitative data on the urinary conjugates of other 17-ketosteroids is scanty. Plantin & Birke (cf. 278) have reported that etiocholanolone was present chiefly as the glucuronoside, whereas androsterone was conjugated with sulfuric and glucuronic acids in approximately equal amounts. Considerable work has been carried out on the nature of the conjugates of the corticosteroids and their metabolites in human urine. Mason (269) reported that the bulk of C_{21} metabolites of endogenous origin was present in a form hydrolyzable by β-glucuronidase (cf., also, 270, 279, 280). A small portion of these substances apparently occurred as sulfates (269). On the other hand, after the oral administration of cortisone acetate, Norymberski (281) found that "sulfate" conjugates accounted for approximately half the urinary content of conjugated 17-ketogenic steroids [17-hydroxycorticosteroids yielding 17-ketosteroids after bismuthate oxidation (272)]. However, in the first few hours after hormone administration, excretion as glucuronic acid complexes predominated (281). Actually, most of the urinary cortisone and cortisol seems to occur free in human urine, although the tetrahydro derivatives of these hormones may be present chiefly as the glucuronosides (cf. 267, 282, 283, 284). The existence of pregnanediol in human urine principally as the glucuronoside has long been recognized. Conditions for the selective hydrolysis of this conjugate with β-glucuronidase have recently been reported [Goldfine & Cohen (285)]. It is apparent from many reports that considerable variation exists in the sensitivity of different steroid glucuronosides to enzymatic cleavage.

Although the importance of the liver in the conjugation of the steroid

hormones appears to be established, available data do not permit a clear picture to be drawn of the nature or extent of hepatic participation in steroid conjugation mechanisms in man and lower animals. West *et al.* (179) reported that liver cirrhosis in human patients did not result in an accumulation of free 17-ketosteroids in blood or urine after the intravenous administration of testosterone. On the other hand, Bongiovanni & Eisenmenger (286) found that individuals suffering from moderate to advanced hepatic disease exhibited an impaired ability to excrete endogenous 17-ketosteroids as the glucuronic acid conjugates. Birke & Plantin (278) suggested that sulfate conjugation of endogenous 17-ketosteroids may also be defective in cirrhotic subjects. An increased proportion of unconjugated formaldehydogenic corticosteroids has been demonstrated in the urine of human patients suffering from hypertension [Dustan *et al.* (287)] and severe pregnancy toxemia [Venning *et al.* (288)]. Glucuronic acid conjugation of metabolites of administered cortisone acetate did not seem to be impaired in Addisonian patients [Burton *et al.* (289)].

West (178) demonstrated that the liver was required for the normally rapid removal of intravenously-injected testosterone from the circulation in the rabbit. However, this phenomenon did not seem to be associated with conjugation of the steroid or its metabolites. *In vitro*, rat liver was reported to be inactive in forming conjugates of testosterone [Samuels *et al.* (290)], corticosteroids [Hechter (291)], or their metabolites. On the other hand, the conjugation *in vitro* of the naturally-occurring estrogens [Szego (223); Crépy (292)] and of stilbestrol [Zimmerberg (293)] in the presence of surviving liver tissue of the rat (223, 293), guinea-pig (292), and rabbit (292) has been successfully demonstrated. Evidence was obtained for the sulfate (293, 294) and glucuronide (223, 292, 293) nature of these complexes.

The relationship of steroid conjugation reactions to the distribution, biological activity, and metabolic fate of this class of hormones has not been defined.

Hydrolysis of Steroid Conjugates in Tissues

Enzymes capable of hydrolyzing steroid conjugates occur widely distributed throughout the body. The relationship of these enzymes to steroid hormone activity and metabolism is as yet obscure. The greatest attention has been paid in this regard to the enzyme, β-glucuronidase, capable of hydrolyzing a wide variety of steroid glucuronosides. The possible relationship of this enzyme to steroid hormone action has recently been reviewed [Fishman (295); Roberts & Szego (4)]. Disagreement exists as to whether its function *in vivo* is primarily hydrolytic or synthetic and whether its role is to make available conjugated or free steroid for action in the target organ. Its activity in certain target organs has been shown to be under the control of circulating steroid hormones. Similar studies do not seem to have been carried out on the distribution and role of sulfatases capable of hydrolyzing the second major class of natural steroid hormone conjugates.

A number of investigations has dealt with the presence in tissues of enzymes capable of hydrolyzing synthetic steroid esters. For example, Bourne (296) examined histochemically the distribution in rat tissues of phosphatases which exhibit a considerable degree of specificity toward estrogen phosphates. Recent work suggests that phosphorylation and dephosphorylation may play an important role in estrogen activity [Diczfalusy (297)] and may, in fact, be involved in the transport of these steroids across the target cell membrane (296). Esterases were demonstrated in blood and other tissues which actively hydrolyze steroid acetates and propionates (298 to 303). These enzymes appear to be essential for the biological activation of administered steroid esters. Thus, Myers *et al.* (cf. 298) observed that the treatment of immature or castrated male rats with tri-*ortho*-cresyl phosphate (TOCP) or triphenyl phosphate (TPhP) suppressed the growth-promoting effect of testosterone propionate on the sex accessories. The action of free testosterone was unaffected. TOCP and TPhP appear to be selective, irreversible inhibitors of ali-esterase activity.

Intermediary Metabolism

Data relating to the intermediary metabolism of the steroid hormones, which have accumulated for almost three decades, continue to be augmented. The principal challenge of this area would appear to be consolidation and systematization of existing information (cf. 1, 57, 304, 305).

Investigations of the participation of nonendocrine organs in steroid transformations are gaining equal prominence with those studies in which end products of over-all steroid metabolism are sought in the excreta. Although the liver plays a dominant role in this regard, the importance of extrahepatic tissues in steroid metabolism in becoming increasingly apparent.

Metabolism of corticosteroids and progesterone in vivo.—Studies of the metabolism *in vivo* of the C_{21} steroids have drawn attention to the multiplicity of possible reactions which these substances may undergo in the normal and diseased organism (cf. 1, 306). The bulk of available evidence indicates that catabolic transformation of the C_{21} steroids is characterized by sequential reduction of the steroid nucleus and its substituents, followed by oxidative splitting of the side-chain [cf. Dorfman (305); Dobriner *et al.* (307)]. Furthermore, the side-chain at C-17 in these steroids may exert a steric influence on the *in vivo* reduction of the unsaturation in ring A, with the resultant predominance of derivatives possessing the pregnane (5β) configuration [cf. Dorfman and co-workers (1, 57, 304, 305)]. Exceptions to these generalizations will be noted below.

The administration of cortisol or cortisone to man has resulted in the urinary excretion of a variety of C_{21} and C_{19} compounds, including appreciable amounts of both of these 17-hydroxycorticosteroids and their tetrahydro derivatives. Classical isolation and identification techniques were utilized in only a portion of these studies. Significant amounts of the un-

changed steroid were recovered in the urine of patients after the administration of cortisone (282, 289, 308, 309) or cortisol (310). The reduction of administered cortisone to urinary cortisol has been confirmed (282, 289, 308, 311). The reverse reaction has now been demonstrated (310, 311). Similar transformations were indicated in the synovial cavity of arthritic patients after the intra-articular injection of cortisone [Wilson *et al.* (312)] or cortisol [Pincus *et al.* (313)]. Several recent investigations have dealt with the further metabolism of these two corticosteroids in the synovium of arthritic patients (312 to 317). The transformation of cortisone to cortisol has also been observed in the presence of hepatic tissue *in vitro* (*vide infra*). Reversible reduction of the 11-keto function in the corticosteroid molecule (cf., also 318, 319) was shown to extend to the interconversion of 11-dehydrocorticosterone and corticosterone *in vivo* [Richardson *et al.* (311)].

A major pathway for the metabolism of the corticosteroids appears to involve hepatic formation of biologically-inactive, ring A-reduced compounds, which are excreted principally as conjugates with glucuronic acid (*vide supra*). Tetrahydrocortisone was first isolated from normal human urine by Schneider (283). This steroid is an important urinary metabolite after the administration of cortisone (282, 289, 308, 309, 311, 320), cortisol (310, 311, 320), or ACTH (cf. 309). The corresponding 11β-tetrol, tetrahydrocortisol (pregnane-3α,11β,17α,21-tetrol-20-one), has also been isolated from the urine of patients given cortisol (310), cortisone (308, 309), or ACTH (cf. 309). Burstein *et al.* detected small amounts of pregnane-3α,17α-diol-11,20-dione, the tetrahydro derivative of 21-desoxycortisone, in human urine following the administration of 21-desoxycortisone (321), but not after cortisone (308), the latter contrary to the observations of Lieberman and co-workers (309). Similarly, the transformation of corticosterone to a number of tetrahydro derivatives has been reported, including pregnane-3α,11β,20α-triol and pregnane-3α,11β-diol-20-one [Fukushima *et al.* (322)], and allotetrahydrocorticosterone (allopregnane-3α,11β,21-triol-20-one) and pregnane-3α,21-diol-11,20-dione [Engel *et al.* (323)]. The last compound (tetrahydro-11-dehydrocorticosterone), as well as its 11β-reduced analogue, tetrahydrocorticosterone, were isolated by Touchstone and co-workers (324) following administration of corticosterone, 11-dehydrocorticosterone, or ACTH. The presence of allotetrahydrocorticosterone and corticosterone was also indicated after ACTH (324). Schneider (284) isolated the dihydro derivative of cortisone (pregnane-17α,21-diol-3,11,20-trione) from normal human urine and suggested that this compound may have been formed by re-oxidation of the C-3 hydroxyl group of tetrahydrocortisone rather than direct reduction of the 3-keto group of cortisone. This conclusion would seem to be supported by the observation of Savard & Goldfaden (318) who tentatively identified dihydrocortisol (pregnane-11β,17α,21-triol-3,20-dione) among the urinary metabolites of orally-administered tetrahydrocortisol in a human patient. However, in the same study, administration of dihydrocortisol resulted in the excretion of the tetrahydro derivatives of cortisol

and cortisone. Along similar lines, Ungar *et al.* (325) reported the isolation of pregnanetriol and small amounts of tetrahydro-17-hydroxy DOC (pregnane-3α,17α,21-triol-20-one) following the administration of the dihydro analogue of the latter compound. None of the parent compound could be detected. The sequence of formation of the dihydro and tetrahydro derivatives of the corticosteroids remains to be elucidated. On the other hand, it appears that reduction of the Δ^4 function in ring A, in C_{19} as well as C_{21} steroids, cannot be reversed *in vivo* (cf. 318, 325).

The conversion of progesterone (cf. 326) and DOC to urinary pregnanediol is well documented. The latter reaction has recently been substantiated by the recovery of radioactive pregnanediol in the urine following injection of DOC-21-C^{14} into the estrous rabbit (47). The isolation of pregnanetriol from normal human urine [Cox & Marrian (327); Bongiovanni & Clayton (328)], and its newly demonstrated origin from dihydro-17-hydroxy DOC (325), suggest that the 17-hydroxylated adrenal precursors of cortisol (i.e., 17-hydroxyprogesterone or 17-hydroxy DOC) may undergo similar transformations. While we have emphasized the metabolic significance of ring A reductions, it may be noted that examples of the occurrence of side-chain reduction (cf. 1, 329) have been extended (47, 309, 322, 325).

As noted above, reduction of the Δ^4-3-ketone function in ring A of the C_{21} steroids appears to result principally in the formation of derivatives of the normal (pregnane) series (cf. 305). However, a number of allopregnane (5α) compounds, some hitherto unknown, have recently been isolated from human urine, including allopregnane-3β,6α-diol-20-one (330) and allopregnane-3β,16α,20α-triol (331) during pregnancy, and allopregnane-3α,17α-diol-20-one in adrenal hyperplasia (322). Likewise, allotetrahydrocorticosterone was the chief ketonic metabolite isolated from the urine of a male, rheumatoid arthritic patient receiving corticosterone (323). It was also detected in the urine of a normal female receiving ACTH intravenously (324). The occurrence of ring D unsaturated compounds in human urine has been noted, e.g., Δ^{16}-allopregnene-3β,6α-diol-20-one (330) and Δ^{16}-pregnene-3α-ol-20-one (322). The metabolic significance of steroid hydroxylations at C-6 and C-16, respectively, has been discussed by Burstein & Dorfman (332) and Lieberman *et al.* (331). The former isolated 6β-hydroxycortisol from human urine during late pregnancy and after the intravenous administration of cortisol to a patient with Cushing's disease following surgical removal of the adenomatous adrenal (333). This steroid has also been isolated from the urine of normal and scorbutic guinea-pigs (334). Oral administration of cortisol to the guinea-pig yielded 6β-hydroxycortisol and, in addition, Δ^4-pregnene-11β, 17α,20α,21-tetrol-3-one and its 20β epimer (332). The survival of the Δ^4-3-ketone group in the principal metabolites of cortisol in the guinea-pig suggests that this species may differ from the human wherein reduction of ring A unsaturation appears to precede alterations or removal of the side chain. However, in this connection de Courcy *et al.* (267) have presented provisional evidence for the occurrence in human urine of two Δ^4-3-ketones (viz., Δ^4-

pregnene-17α,20β,21-triol-3,11-dione and Δ^4-pregnene-11β,17α,20β-triol-3-one), presumably derived from cortisone and cortisol, respectively [cf., also Gray & Lunnon (282)]. Endogenous production of the latter triol has been suppressed after the administration of cortisone to two patients (282). Similarly, cortisone treatment reduced the characteristically high excretion of pregnanetriol in patients afflicted with the adrenogenital syndrome (328, 335).

Available evidence indicates that the urinary Δ^5-3β-hydroxysteroids, including dehydroepiandrosterone, arise from C_{21} adrenal steroids (cf. 3, 278, 336). Lieberman & Teich (337) proposed that pregnenolone may serve as the precursor for dehydroepiandrosterone and related compounds, as well as for the corticosteroids. Oxidative splitting of the side-chain at C-17 in the C_{21} steroids appears to result principally in C_{19} urinary metabolites of the etiocholane (5β) series (cf. 305). However, a number of exceptions to this generalization have been noted (*vide infra*). Only those C_{21} steroids which possess the 17-hydroxyl group appear to be transformed to 17-ketosteroids (cf. 1), although metabolic elimination of the 17-hydroxyl group without concomitant rupture of the side-chain has now been demonstrated (338). Etiocholanolone was found to be a major urinary metabolite of 17-hydroxy DOC (244, 339) and its dihydro analogue (325) in human patients. By the same token, administration of 11-oxy, 17-hydroxy C_{21} steroids has resulted principally in the excretion of 11-oxygenated derivatives of etiocholanolone (*vide infra*), demonstrating the remarkable persistence of the 11-oxygen function during metabolism (cf. 307). In fact, suppression of endogenous production of the 11-desoxy-17-ketosteroids, etiocholanolone and androsterone, has been noted after the administration of cortisone (cf. 278, 308), cortisol (310), dihydrocortisol (318), and tetrahydrocortisol (318), but not after 21-desoxycortisone (321). The major urinary 17-ketosteroid metabolites of the 11-oxy, 17-hydroxycorticosteroids appear to be etiocholane-3α,11β-diol-17-one and etiocholane-3α-ol-11,17-dione. The former conversion product has been demonstrated in the urine of patients given cortisone (cf. 278, 308, 339), cortisol (310, 339), dihydrocortisol (318), and tetrahydrocortisol (318), and in one of two patients after 21-desoxycortisone (321). Similarly, the 11-keto analogue has been found after the administration of cortisone (cf. 278, 308, 339), cortisol (310, 339), dihydrocortisone (325), dihydrocortisol (318), tetrahydrocortisol (318), and 21-desoxycortisone (321). In addition, the $\Delta^{9(11)}$-dehydration artifact of the 11β-hydroxy metabolite, formed during hot acid hydrolysis of the urine (cf. 319), has frequently been noted (278, 308, 310, 318, 321). ACTH administration was reported to result in the increased excretion of 11-desoxy, as well as 11-oxy,17-ketosteroids (cf. 307, 340).

Although the major urinary 17-ketosteroid metabolites of the C_{21} steroids possess the 5β configuration, reservations have been voiced (341) regarding conclusions drawn (cf. 57) from the relatively minor recoveries of 17-ketosteroids in such studies. Moreover, appreciable amounts of 5α (androstane)

derivatives have been demonstrated in some instances. Thus, the excretion of 11β-hydroxyandrosterone was elevated after the administration of cortisone (340), cortisol (341), or 21-desoxycortisol (338) to human subjects. Similarly, androstane-3β,11β-diol-17-one has been isolated from the urine of patients receiving cortisol (342). However, other workers have reported the suppression of endogenous excretion of 11β-hydroxyandrosterone under similar circumstances (310, 318, 321).

Considerable evidence is available from both *in vivo* and *in vitro* experiments to implicate the liver as a major site of corticosteroid catabolism. Thus, the endogenous excretion of 17-ketosteroids has frequently been shown to be depressed in patients with hepatic cirrhosis (cf. 278, 343, 344), even though urinary levels of corticoids were normal or even elevated (343). Similarly, the conversion of administered cortisone (278, 345) or 17-hydroxy DOC (345) to urinary 17-ketosteroids was reduced in patients with cirrhosis (278, 345) or hepatitis (345). Schedl *et al.* (343) noted that compounds which retained the Δ^4-3-ketone function comprised an unusually high proportion of the corticoids excreted by cirrhotic patients. Del Greco *et al.* (346) reported that partial hepatectomy in the rat increased the excretion of formaldehydogenic corticosteroids following oral administration of 17-hydroxy C_{21} steroids. These experiments seem difficult to interpret in view of the fact that 17-ketosteroid elimination was unimpaired. Bacchus and co-workers have suggested that ascorbic acid may inhibit oxidative scission of the corticosteroid side-chain in the liver. They observed that ascorbic acid pretreatment of adrenalectomized rats injected with cortisone prolonged the hematologic action of the hormone (347), promoted the maintenance of the high levels of circulating 17-hydroxycorticosteroids (347), and significantly depressed the cortisone-induced rise in urinary 17-ketosteroids, while enhancing the excretion of the reducing lipide fraction containing corticosteroids (348). Conflicting data pertaining to the possible role of ascorbic acid in steroid metabolism have been published (cf. 91, 145, 334, 349, 350).

Metabolism of corticosteroids and progesterone in vitro.—Hepatic tissue is capable of catalyzing a myriad of transformations and degradations of the C_{21} steroids. More data are available on reductive processes which alter the steroid nucleus or its substituents than on oxidative changes. Much of the metabolic information obtained from *in vitro* studies appears to have its counterpart *in vivo*, but some important differences have been noted.

The reduction of cortisone to cortisol, also demonstrated *in vivo*, has been found to proceed readily in the presence of the isolated, perfused rat liver (351, 352, 353), rat liver slices (354), and homogenates of rat, hog, and beef liver (301, 355, 356). Isolation and characterization of the product was achieved by Caspi *et al.* (353) and by Fish and co-workers (355). The enzymic mechanism appeared to be associated with the microsomal fraction, and not the mitochondria, of liver homogenates [Amelung *et al.* (356, 357)]. The conversion of cortisone to cortisol was reported to be oxygen-dependent and facilitated by the addition of DPN, Mg^{++}, and ascorbic, citric, and succinic

acids (cf. 356, 357). Liver homogenates obtained from male rats were more active than those from female rats (358). The possible significance of this transformation in mediating the physiological effects of cortisone has been the subject of considerable speculation (289, 352, 354, 359). The reverse reaction, cortisol to cortisone, has not yet been demonstrated *in vitro* (cf. 351).

Hechter and co-workers (353, 359, 360) have initiated a detailed study of the metabolism of the corticosteroids in isolated perfused rat livers. A series of spot tests carried out on paper chromatograms of the perfusates revealed a similar pattern of metabolism for cortisone, cortisol, DOC, and 17-hydroxy DOC (359). The major transformations observed involved reductive loss of the Δ^4-3-ketone group (cf., also, 351), in some instances accompanied by modification or cleavage of the α-ketol side-chain. These findings were essentially confirmatory of the studies of Schneider & Horstman (361, 362) employing rat liver slices. Hechter *et al.* (353, 359) detected at least 20 steroid products, most of which were more polar than the original substrate, following the multiple-cycle perfusion of cortisone or DOC through a battery of rat livers. Ten of the cortisone metabolites have been isolated and unequivocally characterized (353, 360). Thus, cortisone perfusion resulted in the formation of 14 α-ketols (including allopregnane-3α,17α,21-triol-11,20-dione, allopregnane-3α,11β,17α,21-tetrol-20-one, the two corresponding 3β epimers, and cortisol), 3 glycols (Δ^4-pregnene-17α,20β,21-triol-3,11-dione, Δ^4-pregnene-11β,17α,20β,21-tetrol-3-one, and allopregnane-3β,17α,20β,21-tetrol-11-one), and 3, 17-ketosteroids (including adrenosterone and androstane-3α-ol-11,17-dione). From these and other investigations (*vide infra*), a number of deductions may be made regarding the stereochemical specificity of C_{21} steroid hydrogenations *in vitro*. For example, reduction of the ketone groups at positions 11 or 20 of the cortisone molecule appears to produce β-hydroxylated derivatives exclusively. Hydrogenation of the C-20 ketone was similarly directed after perfusion of cortisol through the rat liver (360), or incubation of 17-hydroxy DOC with rat liver homogenate [Forchielli (363)]. It will also be noted that reduction of the Δ^4-3-ketone group of cortisone resulted in 3α or 3β hydroxysteroids possessing the allopregnane (5α) configuration exclusively (353, 360). Similarly, allopregnane but not pregnane derivatives have been detected after incubation of DOC (364) or progesterone [Taylor (365)] with rat liver preparations. However, Horwitt & Segaloff (366) reported the isolation of pregnanediol following incubation of progesterone with rabbit liver slices. It will be recalled that C_{21} metabolites of the normal (pregnane) series predominated in the urine in *in vivo* investigations (*vide supra*). Formation of both epimeric alcohols has been observed upon reduction of the 3-ketone group in cortisone by the perfused rat liver (353, 360) and after incubation of DOC with rat liver slices (364). However, only the 3α epimer (allopregnane-3α-ol-20-one) was detected following incubation of progesterone (365) with rat liver homogenates. Under similar conditions, dihydro-17-hydroxy DOC yielded predominantly the 3α-tetra-

hydro derivative [Ungar & Dorfman (367)], whereas pregnane-3,20-dione was reduced mainly to the 3β configuration (368). Thus, it would appear evident that the factors determining the steric direction of reductions in the C_{21} steroid molecule remain to be elucidated. The *in vitro* observations suggest that a multiplicity of enzymatic mechanisms exists in hepatic tissue, some of which may be inoperative or masked (e.g., by further degradation of the steroid molecule) under *in vivo* circumstances, or may make their appearance only under abnormal conditions. In the latter connection, Miller & Axelrod (352) reported the presence of "abnormal" metabolites of cortisone in perfusates of the cirrhotic rat liver (viz., 6β-hydroxy DOC and allopregnane-17α,21-diol-3,11,20-trione). The appearance of these compounds was associated with a decreased capacity of this preparation to effect reduction of the 11-ketone to the 11-hydroxyl group and destruction of the α,β unsaturation in ring A of cortisone, as compared with the normal liver (cf. 351, 353).

Studies have been made of the properties of the enzymatic mechanisms involved in the destruction of the Δ^4-3-ketone grouping in the C_{21} steroids. This reaction has been demonstrated in liver homogenates, as well as in surviving hepatic tissue. It appears likely that more than one enzyme system is capable of catalyzing the reductive process. The ability of homogenates of rat or rabbit tissue to effect the destruction of the conjugated system in progesterone under anaerobic conditions was demonstrated by Wiswell & Samuels (369). Neither a source of high-energy phosphate bonds (e.g. ATP) nor DPN was required, but the reaction was accelerated by the presence of citrate and other tricarboxylic acids. The suggestion was made that the latter promoted the metabolism of progesterone by forming complexes with metal ions, since cysteine and cyanide had a parallel effect. Similar investigations by Taylor (365) confirmed the above observations, except that DPN and nicotinamide were found to exert a stimulatory effect. Taylor (365) isolated from large-scale incubations the reduction products allopregnane-3,20-dione and allopregnane-3α-ol-20-one, as well as the substrate progesterone. The corresponding diols were not detected, nor was pregnanediol (cf., however, 366). The enzyme system responsible for catalyzing the reduction of the Δ^4-3-ketone group in progesterone appears to differ from those which promote similar reactions in the corticosteroids (361, 370) and androgens (290). The latter two required oxygen for their maximal activity. It should be added, however, that Isselbacher & Tomkins (371) reported the presence in liver homogenates of a TPNH-linked enzyme which was capable of destroying with equal facility the α,β unsaturation in ring A of progesterone, testosterone, adrenosterone, cortisone, cortisol, and DOC. The activity of this enzyme system was favored by anaerobiosis. The presence of a DPN-linked 3α-hydrogenase system in liver homogenates was noted by Ungar & Dorfman (367, 368).

The existence of hydroxylating mechanisms in hepatic tissue, similar to those implicated in biosynthetic processes in the adrenal cortex, has been

reported. However, these results cannot as yet be regarded as unequivocal, as the steroid products have been identified only by their behavior on paper chromatograms. Presumptive evidence has been advanced for the 11β-hydroxylation of DOC and 17-hydroxy DOC by various types of liver preparations (126, 141, 301, 351, 357, 358). However, Hübener (372) isolated the product formed upon incubation of 17-hydroxy DOC with bovine liver homogenates and demonstrated that it was similar to, but not identical with, cortisol. The contention of Axelrod & Miller (351, 373) that 17- and 6β-hydroxylating systems occur in liver tissue requires confirmation by direct isolation of the products. Attention must also be given to the possibility of autoöxidation of the steroid molecule in synthetic media (119, 291, 374, 375) and in the blood perfusates (291, 359) employed in these studies.

Oxidative cleavage of the α-ketol side-chain in the corticosteroids has been observed repeatedly in the presence of various liver preparations (351 353, 355, 359, 361, 362, 370, 371). Unequivocal demonstration of this phenomenon was recently provided by Caspi *et al.* who isolated adrenosterone (353) and androstane-3α-ol-11, 17-dione (360) following perfusion of cortisone through the isolated rat liver. Evidence was also obtained in these studies for the formation of one other 17-ketosteroid (353). Cirrhotic liver preparations exhibited curtailed capacity for side-chain cleavage (352). Progesterone appears to be resistant to loss of the side-chain in the presence of liver homogenates (369). Bacchus and co-workers (347, 348, 350, 370, 376) reported that ascorbic acid exerted an inhibitory influence on the loss of the side-chain and other transformations of the corticosteroid molecule. Evidence supporting this contention was obtained from studies carried out *in vitro*, as well as *in vivo* (*vide supra*). Thus, liver slices from rats or vitamin C-deficient guinea pigs treated with massive doses of ascorbic acid exhibited a reduced capacity to metabolize cortisone and DOC, as compared with similar tissue from untreated animals (370, 376). Pretreatment of the animals with the vitamin protected both the α,β unsaturation in these corticosteroids (as determined by ultraviolet absorption measurements) and the α-ketol side-chain (giving the Porter-Silber or Heard-Sobel reactions). Anaerobiosis, or ascorbate added *in vitro*, depressed the rate of destruction of the Δ^4-3-ketone system, but was without effect on the loss of the side-chain. These studies would gain added significance if the identification procedures were applied to more purified extracts of the incubates (cf. 11, 377, 378). However, the work of Bacchus *et al.* provides additional evidence for the existence in liver tissue of discrete enzymatic mechanisms which attack separate loci in the corticosteroid molecule.

Metabolism of androgens and other C_{19} steroids in vivo.—The urinary C_{19} steroids have long been known to arise from neutral steroid precursors of both the C_{19} and C_{21} configuration. This dual origin has complicated the diligent search for the pathways which participate in the metabolism of the neutral steroid hormones. The major approach to this problem has involved the isolation and characterization of urinary steroids following the

administration of possible steroid precursors. Interpretation of results, even when isotopic steroid precursors have been employed, has been severely limited for a number of reasons (*vide infra*). One of the more serious limitations has been recognized, namely the fact that conditions associated with the hydrolysis and extraction of urine may give rise to steroid artifacts [cf. Teich *et al.* (379)].

In recent years Dorfman and his colleagues (1, 57, 304, 305) have coordinated in a systematic fashion the reactions whereby metabolites of the neutral steroids arise from their precursors *in vivo*. This scheme is a valuable and stimulating synthesis of existing data. However, some of the generalizations proposed regarding the nature and sequence of steroid catabolic mechanisms, and the stereochemical influences on these pathways, are supported by meager data (cf. 380). The proposed scheme is admittedly subject to such modifications as may be demanded with time (57, 305).

An important postulate of Dorfman's unified hypothesis is the presumed regulation exerted by substituents in the basic steroid structure on the sequence of catabolic reactions and the steric nature of the products. Thus, reduction of C_{19} compounds possessing the Δ^4-3-ketone structure in ring A and an additional oxygen at C_{17} (e.g., androstenedione and testosterone) was predicted to proceed at C-4 and C-5 with the formation of approximately equal amounts of metabolites of the 5α (androstane) and 5β (etiocholane) series. Dehydroepiandrosterone may be considered a potential member of this group in view of the ease of transformation of its Δ^5-3β-hydroxy function to the Δ^4-3-ketone in hepatic (381) and steroidogenic (cf. 112) tissues. On the other hand, reduction of analogous compounds possessing an additional oxygen at C-11 (e.g., adrenosterone) would presumably be sterically directed in favor of the 5α configuration. These deductions were based on the wide differences in the ratio of 5α and 5β urinary metabolites following the administration to human subjects of the two classes of compounds (cf. 304, 305, 382). Since the observations were limited to few individuals, some clinically abnormal, it is not surprising that some recent data do not fit the predicted pattern. Thus, the Sloan-Kettering group reported that the urinary ratio of labeled androsterone to etiocholanolone after intramuscular administration of deuterotestosterone was similar to the pre-existing endogenous ratio in the individual (255, 383). In addition, the ratio was observed to change with time, even to the extent of inversion, following the intravenous injection of isotopically-labeled testosterone (181). These investigators suggest that the ratio of the urinary 5α and 5β C_{19} ketosteroids is a reflection of the metabolic pattern characteristic of the individual, rather than of stereochemically directed enzymic reactions (cf. 58, 181, 244, 255, 383, 384).

The interpretation of urinary steroid patterns following administration of steroid precursors is limited by the difficulty in assessing the factors of varying dosage, differences in routes and rates of administration, possible suppression or potentiation of endogenous steroid metabolism, and participation of alternate metabolic pathways for administered steroids. For ex-

ample, the per cent of administered isotope recovered in urinary androsterone and etiocholanolone following the intramuscular injection into men of labeled testosterone was greater when 10 mg. of the steroid was used, rather than 100 mg. (244, 255, 383). The recovery of these 17-ketosteroids after the rapid intravenous infusion of 100 to 150 mg. of testosterone was greater than that following chronic intramuscular injection [cf. West *et al.* (385)]. However, Fukushima *et al.* found that small doses of testosterone (10 to 15 mg.) did not result in significant differences in recovery of androsterone and etiocholanolone after intramuscular (383) or intravenous (181) administration. The experiments of Fukushima *et al.* (181) and of West *et al.* (385) differed further in that the rate of intravenous infusion of testosterone was prolonged (4 to 8 hr.) in the former studies. In the rodent, variation in the dose of intraperitoneally administered testosterone was without obvious effect on the excretion pattern (249, 255). Suppression of endogenous excretion of C_{19} steroids has been demonstrated in human subjects following the administration of either C_{19} (382, 383, 386) or C_{21} (278, 308, 310, 318) compounds. This phenomenon would appear to be minimized by the use of small doses of isotopically labeled materials (383) and of shorter urinary collection periods made possible by rapid intravenous infusion of the steroid (385).

Recent experiments employing isotopic testosterone in man (181, 244, 383) have amply confirmed the concept that this compound is directly metabolized to its principal urinary products (androsterone and etiocholanolone) by oxidation and reduction of functional groups without drastic degradation and regeneration of the steroid skeleton. In these studies (383) small amounts of isotope appeared in the diketones, androstane-3,17-dione and etiocholane-3,17-dione, in 3β-hydroxyketosteroids, and in nonketonic metabolites. The recovery of the diketones, unlike that of androsterone and etiocholanolone, did not appear to be dependent on testosterone dosage. On the basis of these observations, Fukushima *et al.* (383) questioned the general assumption that the diketones are on the direct pathway from testosterone to its principal metabolites (cf. 1, 3, 319, 387) and provisionally suggested that they arise from oxidation of the latter. The analogous problem relating to the sequence of formation of dihydro and tetrahydro derivatives of the corticoids has been discussed above.

In attempts to delineate the metabolic pathways leading to the formation of androsterone and etiocholanolone, and of other urinary C_{19} metabolites of both gonadal and adrenal origin, a wide variety of possible steroid precursors has been administered to human subjects. Androsterone has been demonstrated by chromatographic techniques (386) and isolation procedures (381) to be a urinary metabolite of androstane-17β-ol-3-one in an agonadal male (386) and of Δ^5-androstene-3β,17β-diol in a rheumatoid arthritic man (381). Epiandrosterone was also indicated in the former study (386). The administration of adrenosterone to a 61-year old rheumatoid arthritic man resulted in the excretion principally of 11-oxygenated analogues of androsterone and etiocholanolone [Savard *et al.* (382)]. Urinary metabolites of the androstane

series predominated over those of the etiocholane type. This was in sharp contrast to the earlier demonstration of Dorfman *et al.* (cf. 57) that the administration of androstenedione, the 11-deoxy analogue of adrenosterone, resulted in an approximately equivalent excretion of androsterone and etiocholanolone. Ungar *et al.* (381) isolated etiocholanolone from the urine of a rheumatoid arthritic man and of guinea pigs after the administration of Δ^5-androstene-3β,17β-diol. Dehydroepiandrosterone was an additional metabolite isolated in these studies. The ready conversion of dehydroepiandrosterone to androsterone and etiocholanolone in man has been established (cf. 1, 57). The formation of etiocholanolone from dehydroepiandrosterone has also been demonstrated in the guinea pig (381). Since dehydroepiandrosterone does not arise from testosterone (1, 387), these two steroids probably pass through a common intermediate during their metabolism (cf. 1, 2, 3). This may be androstenedione, which is formed from both of these precursors by hepatic tissue *in vitro* (vide infra).

The lability of androstenedione to further transformation is indicated by the fact that it does not normally occur in urine. However, this steroid (cf. 388) and its 11β-hydroxy analogue (389) have been isolated from the urine of patients with adrenocortical hyperfunction. Other endogenous C_{19} steroids isolated from the urine in similar conditions, confirming or extending previous findings, include Δ^{16}-androstene-3α-ol (388) and Δ^5-androstene-3β-ol-7,17-dione (322). Lieberman *et al.* (331) reported the isolation from human urine of two hitherto-unrecognized 16-hydroxylated steroids: etiocholane-3α, 16α, 17β-triol and androstane-3α, 16α,17β-triol. These were presumed to be oxidation products of etiocholanolone and androsterone, respectively. The 3β-hydroxymonoketoandrostane compound isolated from pregnant mare urine by Heard & McKay (cf. 390) has been identified by Huffman & Lott (390) as androstane-3β-ol-16-one.

Metabolism of androgens and other C_{19} steroids in vitro.—The key position of Δ^4-androstene-3,17-dione as an intermediate in the metabolic transformations of the C_{19} compounds of both testicular and adrenal origin has been re-emphasized by recent investigations *in vitro*. Samuels & West (387) summarized the extensive data relating to the transformation of testosterone to androstenedione and its further degradation in hepatic tissue of many species of animals. The enzyme responsible for the formation of the 17-ketosteroid appears to be a DPN-linked dehydrogenase [Sweat *et al.* (cf. 387)]. Further degradation of the steroid molecule by liver tissue involved reduction of the α, β unsaturated ketone system in ring A (cf. 387). At least two distinct enzymatic activities seemed to be involved in these transformations. Of all tissues studied, only kidney exhibited the 17-oxidizing capacity. The reversibility of this reaction has been demonstrated by Kochakian *et al.* (391, 392) in homogenates of liver and kidney tissue obtained from the rabbit and guinea pig. More recently, Wotiz and co-workers (187, 393, 394) have shown that a wide variety of surviving human tissues were capable of metabolizing added testosterone, in part to a steroid possessing the paper

chromatographic and infrared absorption characteristics of androstenedione. Additional steroid metabolites were also formed but were incompletely characterized (187, 393). Normal skin and mammary carcinoma tissue were exceptionally active in effecting the oxidative transformation; liver tissue was relatively low in activity (187, 394). Human serum was also found to possess activity, which disappeared after boiling (395). Benign hyperplastic and neoplastic prostatic tissue of human origin formed androstenedione from testosterone in significant amounts (187, 393) and appeared to effect further degradations (396). Samuels *et al.* (290) had earlier been unable to demonstrate these transformations in normal prostatic tissue from the rat. These latter data would appear to bear on the fundamental problem of whether changes in the steroid molecule accompany hormonal stimulation of the target organ. Wotiz *et al.* (187, 394) also observed that human testicular and prostatic tissue were capable of reducing androstenedione to testosterone. The equilibrium was predominantly in the oxidative direction in all tissues other than testis. An analogous situation exists with respect to the interconversion of estrone and estradiol in various human tissues (397).

Kochakian & Nall (398) demonstrated that the 17α epimer of testosterone (epitestosterone) was also converted to androstenedione by rabbit liver and kidney preparations *in vitro*. The enzyme involved seemed to be similar to the DPN-linked dehydrogenase responsible for the oxidation of testosterone. The reverse reaction was also demonstrated in rabbit liver and kidney homogenates (392, 399). In the latter experiments androstenedione was converted to testosterone, epitestosterone, androsterone, and androstane-3,17-dione. Analogous conversions occurred in guinea pig liver and kidney homogenates except that the formation of epiandrosterone, but not of epitestosterone, could be demonstrated. Etiocholane and nonketonic metabolites could not be detected in any of these studies. The unique capacity of rabbit tissues to reduce 17-ketosteroids with the formation of the 17α hydroxylated derivative has also been demonstrated for the estrogens (*vide infra*).

The transformation of C^{14}-dehydroepiandrosterone to androstenedione has finally been demonstrated in rat liver perfusates by Ungar *et al.* (381). Samuels *et al.* (see 387) had previously been unable to demonstrate the conversion of the Δ^5-3-hydroxy configuration to the α,β unsaturated ketone in liver slices. The probable adrenal origin of dehydroepiandrosterone has already been noted. Ungar *et al.* (381) also found that rat and rabbit liver preparations transformed dehydroepiandrosterone to Δ^5-androstene-3β,17β-diol, but not to the 16α-triol. The latter conversion had been reported earlier by Schneider & Mason (cf. 381), employing rabbit liver slices.

Further transformations of the 17-ketosteroids *in vitro* have been noted. For example, Axelrod & Miller (373) demonstrated that the perfused rat liver was capable of effecting the 6β-hydroxylation of testosterone and androstenedione. In view of existing possibilities for auto-oxidation (*vide supra*), control perfusions in the absence of liver appear desirable. Bischoff *et al.* (400) have reported the presence of enzymes in erythrocytes which modify

certain functional groups in ketosteroids. The significance of this is obscure.

Metabolism of estrogens in vivo.—Quantitative and qualitative species differences have been observed in the recovery of excreted metabolites of administered estrogens. Pearlman and co-workers (cf. 401) and Gallagher *et al.* (244) measured the recovery of isotope in the estrogenic fraction of human urine after the injection of 6,7-d_2-estrone acetate (401) and of C^{14}-labeled estrone or estradiol-17β (244). Intramuscular injection of relatively large amounts of the deuterium-labeled estrogen (ca. 100 mg.) to three pregnant women resulted in the urinary excretion of about 9 per cent of the administered dose as estrone and estriol (401). Small amounts of isotopic estradiol were also detected (<1 per cent). These recoveries were quantitatively similar to those obtained earlier by Pincus & Pearlman (cf. 401) in nonpregnant women and in men in parallel experiments employing nonisotopic estrone. The above observations would appear to provide evidence against the hypothesis of Smith & Smith (cf. 401) that progesterone facilitates the conversion of estrone to estriol during pregnancy and thereby inhibits the oxidative degradation of estrogen. Gallagher *et al.* (244) reported the recovery of much larger amounts (ca. 25 per cent) of administered C^{14}-labeled estrogens in the ether-soluble phenolic fraction of urine in female subjects. However, in the latter experiments very small amounts (ca. 1.0 mg.) of the isotopic precursors were infused intravenously, and no attempt was made to isolate and purify the metabolites.

Intravenous injection of estrone-16-C^{14} in the pregnant mare resulted in the recovery of about 8 per cent of the administered dose in the urinary strong-phenolic ketones from which estrone of moderately high activity was isolated (47). Significant amounts of radioactivity did not appear in ring B-unsaturated estrogens. The latter observations provide evidence that estrone does not serve as a precursor for equilin and equilenin in the mare. Fractionation of the urinary phenols after subcutaneous administration of estrone-16-C^{14} to a pseudopregnant rabbit revealed radioactivity only in the estradiol fraction [Stimmel (262)]. The latter appeared to be the 17α epimer characteristic of this species. In the mouse, only a small fraction of the administered radioactivity (0.49 per cent) was found in the urinary ether-soluble phenols during the five-day period subsequent to the subcutaneous injection of 1 mg. of estrone-16-C^{14} (47). Axelrod (261) has provided the first evidence for the occurrence of estriol in the excreta of sub-primate species. This steroid was detected in the bile of an ovariectomized, hysterectomized dog after the intramuscular administration of estradiol-17β-dibenzoate. Methods of identification included ultraviolet absorption and sulfuric acid chromogenicity of the biliary metabolites separated by paper chromatography (cf. 11). Pearlman *et al.* (259) had earlier been unable to isolate estriol from the bile of normal dogs after intramuscular injection of massive doses of estrone acetate.

Extensive metabolism of administered estrogens, including degradation of the steroid nucleus, has been reported in experiments employing isotopic compounds (*vide supra*). Under such circumstances radioactivity was de-

tected in the ether-soluble acidic and neutral fractions of urine in the mouse (47), pseudopregnant rabbit (262), and human (244). A high proportion of the radioactivity in the urine and feces of the mouse, even after acid hydrolysis, was in a water-soluble form, including urinary urea (47). In the latter studies, isotope was also detected in the respiratory CO_2 collected over a 100-hour period subsequent to estrogen administration.

Metabolism of estrogens in vitro.—The interconversion of estrone and estradiol, as well as the degradation of these compounds to unrecognized metabolites, has also been demonstrated in the presence of surviving tissues. However, the formation of estriol under these conditions has not been confirmed (cf. 397).

Contradictory evidence has been presented on the capacity of erythrocytes to metabolize estrogens *in vitro*. Bischoff and co-workers have extended their earlier studies of the "estronase" system in rabbit erythrocytes which allegedly transform estrone to a more active estrogen (cf. 402, 403). The reaction product, earlier thought to be some highly-active unknown estrogen (cf. 402), is believed to be estradiol (403). Bischoff *et al.* (402) noted the widespread distribution of "estronase" activity in the tissues of rodents and man. However, the properties of the enzyme appeared to vary in different tissues. The ability of tissues other than blood to effect the reduction of estrone to estradiol seems well established from earlier investigations. In addition, Werthessen *et al.* (cf. 404) have reported that normal human erythrocytes were capable of reducing the biological activity of added estrone under certain conditions *in vitro*. This phenomenon appeared to be associated with loss of the 17-ketonic group of the estrogen, as judged by polarographic analysis. In an effort to resolve this problem of estrone activation or inactivation by erythrocytes, Levy (404) applied chromatographic analysis to large-scale incubates of estrone in human blood, provided by the Werthessen group. Almost quantitative recovery of the estrone initially added was achieved. In one experiment in which estrone-16-C^{14} was the substrate, no radioactivity was found in the "estradiol" fraction. On the basis of these results the observations of Bischoff *et al.* and Werthessen and co-workers, in which changes in biological activity and polarographic properties were the criteria employed, would appear to require re-evaluation.

The capacity of liver and other tissues to metabolize estrogens has been reinvestigated by Ryan & Engel (397, 405), employing the newer techniques of counter-current fractionation and photofluorometric estimation of the products. Rat liver slices effected the interconversion of estrone and estradiol under anaerobic as well as aerobic conditions (405). In agreement with earlier studies from other laboratories degradation of these steroids was diminished when oxygen was excluded from the incubation mixture. It should be noted that the extraction methods employed would not have permitted recognition of the aerobic formation of conjugates such as glucuronosides, observed by others (223, 292) under similar circumstances. Some evidence was presented for the reduction of estrogen metabolism in hepatoma and cirrhotic liver (cf.,

also, 222). In related studies Ryan & Engel (397) demonstrated that a variety of human operative tissue specimens, many grossly abnormal, catalyzed the interconversion *in vitro* of estrone to estradiol and degraded these steroids to unrecognized metabolites. The oxidative reaction predominated in all tissues studied with the exception of testis. Testicular tissue has also been reported to be exceptionally high in "estronase" activity (402) and to be capable of catalyzing the reductive conversion of androstenedione to testosterone, but not the reverse reaction (187, 394). Ryan & Engel (397) reported that estriol was not formed from estrone or estradiol in the presence of surviving human tissues. However, as noted above, conjugates of estriol or the other estrogens would not have been detected in these experiments. Samples of human feces have also been reported to reduce estrone-16-C^{14} to estradiol (406).

The capacity of rat liver preparations to catalyze the binding of estrogenic steroids to proteins in the incubation medium has been discussed (*vide supra*). As noted earlier, the binding of estrogens to the serum and liver proteins may represent two distinct phenomena, the first related to the transport and activity of this class of steroids (cf. 4) and the second associated with their metabolic inactivation (cf. 225).

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BIOCHEMISTRY OF ANTIBIOTICS[1,2]

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In preparing a review of this type, it is necessary to set an objective and choose the material that is pertinent to attain this end. Otherwise the only accomplishment would be a cataloguing of the many references in the field. Accordingly, this review will be concerned primarily with the chemistry of the antibiotics with the hope that we will be able to correlate chemical structure with activity and thereby gain some understanding as to how a group of compounds of such diversified structures can prevent growth. Having decided on this objective, a review and evaluation of the biochemical changes brought about by these compounds in microorganisms will be necessary. Related fields such as bacteriology, nutrition, and clinical application will be considered only if they contribute to the problem. There are several review articles on these subjects (1 to 7).

In past reviews the antibiotics have been classified on the basis of elementary composition (8, 9), and on the basis of the organisms producing them (10, 11). Since closely related antibiotics (the tetracyclines) would fall into different groups under the first method, and since most of the antibiotics studied in the past two years have been produced by the *Streptomyces*, both methods lose their usefulness. A more desirable method would be on the basis of biochemical functions of the compounds. Because of the paucity of information in this field, the best that can be done is a modification of this latter type of classification. The antibiotics, therefore, will be classed into (*a*) those active against gram-positive organisms, (*b*) the broad spectrum antibiotics, (*c*) the streptothricin-like compounds, (*d*) the polypeptides, (*e*) those active against fungi, and (*f*) a miscellaneous group. In some cases an antibiotic will be included in one group because of its similarity in chemical structure to the antibiotics under discussion.

Of outstanding interest during the period covered by this review has been the chemical study and clinical application of the tetracycline group of antibiotics. Azaserine and puromycin, antibiotics having inhibitory activity against experimental tumors, have been synthesized. Erythromycin and carbomycin, active against gram-positive organisms, are in commerical use, and several papers reporting on their chemistry have appeared. A long list of new antibiotics, many of which are poorly characterized, has been reported. The work of Parke on the isolation of a nucleotide containing a derivative of a hexosamine from penicillin inhibited cells has led to a biochemical study of this new class of interesting compounds.

[1] The survey of the literature pertaining to this review was completed in October, 1954.

[2] The following abbreviation is used in this review: DNP for dinitrophenyl.

Antibiotics Active Against Gram-Positive Microorganisms

Penicillin.—Abraham *et al.* (12) have recently described a method for the purification of cephalosporin N, an antibiotic having many of the properties of a penicillin, but differing strikingly in its antibacterial activity (13, 14) and hydrophilic character. Because of the inability to extract the compound into organic solvents, purification by chromatography on charcoal and alumina and by countercurrent distribution was necessary. Degradative studies (15, 16) showed the antibiotic to be (D-4-amino-4-carboxy-*n*-butyl)-penicillin. Cephalosporin N reacts with 1,2,4-fluorodinitrobenzene, benzoylchloride, benzyloxycarbonyl chloride, phenylisocyanate, and acetic anhydride to form derivatives which contain no free amino group. These derivatives are more active against *Staphylococcus aureus* and less active against *Salmonella typhi* than cephalosporin N itself.

Synnematin A and B (17 to 20), produced by an organism similar to that producing cephalosporin N, may be closely related to this antibiotic. Solubilities, stabilities, and antibacterial activities are very similar. The action of penicillinase on synnematin A and B has not been reported.

By using *p*-aminophenylacetic acid as a precursor in penicillin fermentation, Brewer & Johnson (21) prepared *p*-aminobenzylpenicillin. It was shown to be excreted at one-third the rate of penicillin G and consequently to maintain higher blood levels. It was somewhat more active than penicillin G.

The search for insoluble salts of penicillin which are clinically useful has continued. The one other than procaine penicillin that has received most widespread use is dibenzylethylenediamine dipenicillin salt (22, 23, 24).

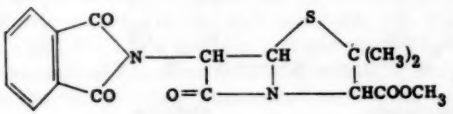
The reaction of triethylammonium benzylpenicillinate with ethyl chloroformate has provided a mixed anhydride which has served for the preparation of amides, esters, and thiolesters of penicillin (25, 26).

Sheehan *et al.* (27, 28) have continued their work on the synthesis of penicillin and have succeeded in preparing a β-lactam-thiazolidine, I, which has the complete structure (configuration unassigned) of the natural penicillins, except for the substitution of a phthalimido group for the acylamino side chain. The key intermediate is a penicilloic acid, II, which is incapable of azlactonization. This compound is biologically inactive by *in vitro* screening methods.

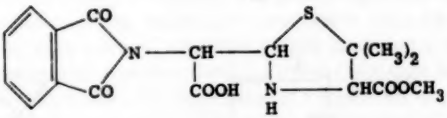
Treatment of benzylpenicilloic acid or residues from the commercial production of penicillin with acetic anhydride and pyridine gave 2-keto-4-acetyl-3,4,5,4′,3′,2′-(5′,5′-dimethyl-thiazoliod)-piperazine which was synthesized (29). An inactive by-product of penicillin production, sorbicillin, was shown to be 2,4-dihydroxy-3,5-dimethylsorbophenone (30). DL-Penicillamine was condensed with methyl *n*-γ-formylbutyrate to give an analogue of penicillin (31).

In a study of the biosynthesis of penicillin it was shown that no significant amount of carbonate-C^{14} was incorporated while formate-C^{14}, acetate-1-C^{14}, and acetate-2-C^{14} were incorporated. Lactate-1-C^{14} was incorporated to a lesser degree (32). The carbonyl of the β-lactam ring originates from the

carboxyl of acetic acid, and the methine group attached to the carbonyl appears to arise from the methyl group of acetate. Carbon-C^{14} from acetate was also incorporated into the penicillamine portion of the molecule (33). A study of the incorporation of phenylacetic acid-1-C^{14} into benzylpenicillin showed that phenylacetic acid isolated by hydrolysis of penicillin contained all the label in the carboxyl group (34). The same workers also showed a rough correspondence between the specific activity of the respiratory CO_2 and that of the CO_2 of the β-lactam carbonyl pointing to the possibility of CO_2 fixation playing a role in penicillin biosynthesis (35). L-Cysteine, L-cystine, DL-methionine, and glutathione were utilized as a source of sulfur in preference to inorganic sulfate (36). The high yield of methionine S_{35}



I



II

from sulfate in penicillin fermentations is considered to indicate an important role of methionine in the biosynthesis of penicillin (37).

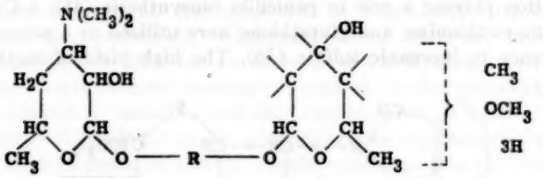
In an outstanding piece of work Arnstein & Grant (38, 39) showed that the penicillamine portion of the molecule was derived from valine and using a triple labeled molecule, that the nitrogen, β-carbon, and sulfur atoms of cysteine were incorporated with unchanged isotope ratios into the thiazolidine and β-lactam rings of penicillin. However, Stevens *et al.* (40) have shown that β-(L-2-amino-2-carboxyethylmercapto)-D-valine is not an effective precursor of penicillin sulfur.

Erythromycin.—Erythromycin is an antibiotic produced by a strain of *Streptomyces erythreus* (41) and active against gram-positive bacteria and fastidious gram-negative organisms. It is a crystalline colorless compound slightly soluble in water but dissolves readily in most organic solvents (42, 43, 44). Crystallographic and molecular weight data are reported by Rose (45). Erythromycin is a base, having a pK_a' of 8.6, a molecular extinction of 27 at 278mμ, and a molecular formula of $C_{37}H_{67-69}NO_{13}$.

Mild acid hydrolysis yields two compounds, one a base, $C_{29}H_{49}NO_8$, melting at 207–208°C. and the other a liquid, $C_8H_{16}O_4$ (cladinose, III).

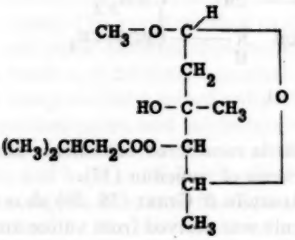
Strong acid hydrolysis of the base gives propionaldehyde and desosamine, $C_8H_{17}NO_3$. The structures of desosamine and cladinose have been partially determined and a partial structure, (III), proposed for erythromycin.

A second antibiotic, erythromycin B, has been isolated from culture filtrates of *S. erythreus* (46, 47). The compound gives the analysis for $C_{37}H_{71}NO_{12}$. On mild acid hydrolysis cladinose and two crystalline bases $C_{29}H_{55}NO_9$, m.p. 119–121°C., and $C_{29}H_{51}NO_8$, m.p. 239–240°C., were iso-

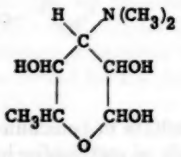


$R = C_{21}H_{36-38}O_6$

III



IV



V

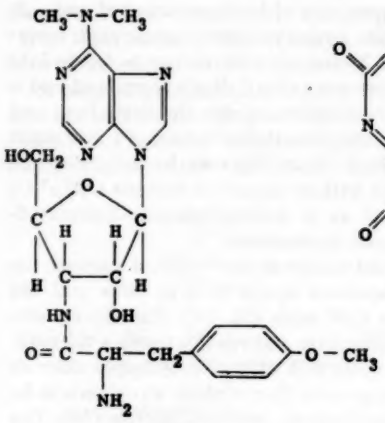
lated. The higher melting base probably represents a dehydration or oxidation product of the lower melting base. Strong acid hydrolysis of the lower melting base yielded desosamine and a volatile α,β-unsaturated aldehyde or ketone containing six carbon atoms. No propionaldehyde was formed as was the case with erythromycin. Physical data also show differences in the "central" portion of the molecule.

Carbomycin.—Carbomycin is an antibiotic produced by *Streptomyces halstedii* and is particularly active against *Richettsia* (48, 49, 50) and gram-positive organisms. The colorless crystalline base shows strong absorption at 238 mμ ($E_{1cm}^{1\%} = 185$) and weak absorption at 327 mμ ($E_{1cm}^{1\%} = 0.9$). Analyses show an empirical formula of $C_{41-42}H_{67-69}NO_{16}$ (51, 52). Chemical

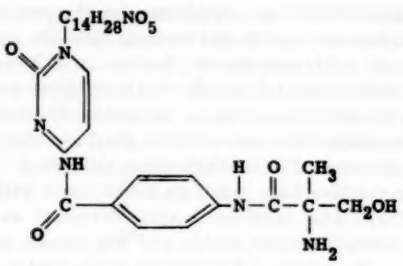
and physical properties suggest the presence of both unsaturated and α,β-unsaturated carbonyl systems. Alkaline hydrolysis yields acetic acid, isovaleric acid, and dimethylamine. Acid hydrolysis cleaves the molecule into two fragments, namely, the isovalerate ester of a $C_7H_{14}O_4$ compound and a crystalline base, $C_{29-30}H_{47-49}NO_{12}$. Methanolysis yields the same base and a neutral substance which is the 4-isovaleryl methyl glycoside of a new sugar mycarose, IV, (configuration unknown). More vigorous hydrolysis of the crystalline base yields an amino sugar with an empirical formula $C_8H_{17}NO_4$ (53). The compound was identified as a 3-dimethylamino-3-desoxy-5-methylpentopyranoside and was named mycaminose, V.

Puromycin.—Puromycin is a broad-spectrum antibiotic of interest because of its activity against *Trypanosoma equiperdum* in mice and the transplanted adenocarcinoma of the C_3H mice (54, 55). Puromycin, empirical formula $C_{22}H_{29}N_7O_5$, is a diacidic base and readily forms a dihydrochloride or a monosulfate. On alcoholysis with ethanolic hydrogen chloride the antibiotic is cleaved into three fragments. One of these was shown to be 6-dimethylaminopurine by comparison with an authentic sample (56). The second fragment was identified as O-methyl-L-tyrosine. The third fragment was isolated as its hydrochloride, $C_8H_{11}NO_4 \cdot HCl$. It shows a positive Fehling's test, a positive Brady's reaction, a negative ninhydrin test, and forms furfural on deamination and subsequent treatment with phosphoric acid. These data along with infrared absorption data indicated a 3- or 4-aminopentose. The compound was identified as D-3-aminoribose by comparison with a synthetic sample (57). The formation of a triacetate of puromycin and subsequent partial deacetylation to N-acetylpuromycin indicates two free alcoholic groups. The free amino group in puromycin is placed in the O-methyltyrosine moiety by the failure of puromycin to consume periodic acid. This failure to consume periodate also eliminates a 4-aminopentose structure for the aminopentose. A negative Brady's test until after hydrolysis indicates a glycosidic linkage in the antibiotic. A study of ultraviolet absorption spectra established the linkage at the 9 position of the purine. The β-nature of the glycosidic linkage and the furanosidic structure were established by removal of the O-methyltyrosine moiety by the thiohydantoin method to give 6-dimethylamino-9-(3′-desoxy-3′-aminoribosyl)-purine. Uptake of one mole of periodate by this compound establishes the furanose structure. A comparison of the dialdehyde with the same dialdehyde from 6-dimethylamino-9-β-D-glucopyranosylpurine established the β-linkage between the carbohydrate moiety and the purine. The complete structure of puromycin is 6-dimethylamino-9-[3′-desoxy-3′-(*p*-methoxy-L-phenylalanylamino)-β-D-ribofuranosyl]-purine, VI (58).

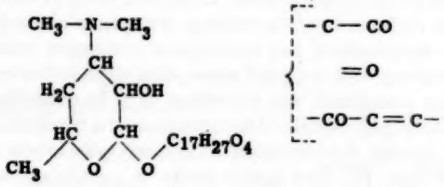
Unfortunately, the space allotted to this review is not sufficient to permit a detailed review of the outstanding work of Baker *et al.* (59 to 65) on the synthesis of puromycin. The starting carbohydrate was methyl-D-xylofuranoside which was converted to methyl 2,3-anhydro-D-lyxofuranoside and the ring opened with ammonia to give methyl 3-amino-desoxy-D-



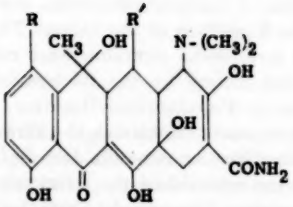
VI



VII



VIII



IX R = Cl, R′ = H

X R = H, R′ = OH

XI R = H, R′ = H

arabinofuranoside. Walden inversion by means of the mesylate gave the desired substituted ribose. Thus, in this synthesis all four of the stereoisomeric pentoses were utilized.

The high activity and difference in bacterial spectra of 6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)-purine is of particular interest (55). The "aminonucleoside" has lost most of its antibacterial activity, but the activity against *T. equiperdum* was increased three- to four-fold. Puromycin

has a medium order of activity against the transplanted mammary adenocarcinoma of the C_3H mouse, the "aminonucleoside" being highly effective against this tumor. The corresponding "aminonucleoside" having an α-glycosidic linkage in place of the β-linkage is inactive. When the O-methyltyrosine moiety in puromycin was replaced with other amino acids, the compounds were active (55).

Griseomycin (formerly lomycin).—Griseomycin is a new antibiotic produced by a *Streptomyces* closely related to *Streptomyces griseolus* (66). Solubility, infrared spectra, biological activity, cross resistance, and pharmacological studies indicate that the antibiotic is closely related to erythromycin and carbomycin. Since its physical constants are different from those of either carbomycin or erythromycin, it is likely that this antibiotic is new.

Amicetin.—Amicetin is an antibiotic produced by *Streptomyces fasiculatus* and active mainly against acid-fast and gram-positive organisms (67, 68). Its toxicity varies with the species. The pure antibiotic is a basic compound with a pK_a of about 7. Titration data and analyses indicate a formula of $C_{29}H_{44}N_6O_9$ for the free base (69). Hydrolysis experiments showed the presence of cytosine, *p*-aminobenzoic acid, and D-α-methylserine in the molecule (70). On the basis of these fragments and a study of other hydrollysis products containing them, structure VII was proposed for amicetin.

Picromycin.—Brockmann *et al.* (71, 72, 73) have isolated and studied an antibiotic named picromycin, obtained from the culture filtrates of an *Actinomycete.* Many of its properties are similar to those of erythromycin. Analytical data indicate a formula of $C_{25}H_{43}O_7N$. Hydrolysis yields cromycin, $C_{16}H_{24}O_4$, and picrocin, a dimethylamino sugar. All the evidence indicates that the latter substance is identical with desosamine isolated from erythromycin. A partial structure, VIII, of the antibiotic has been proposed.

Phalamycin.—This antibiotic is produced by a *Streptomyces noursei* variant, the same organism that produces fungicidin which is fungicidal and not antibacterial (74). It is active against gram-positive bacteria and *Mycobacterium tuberculosis.* It is not highly toxic and appears to have *in vivo* activity. The antibiotic is a nitrogen containing neutral compound, soluble in the common organic solvents and sparingly soluble in water. It decolorizes bromine and shows a slight Benedict's test. Biuret, ninhydrin, Sakaguski, Molisch, and Millon's tests are negative. Both primary and secondary alcoholic groups are present.

Streptogramin.—Streptogramin, an antibiotic active against gram-positive organisms, is produced by *Streptomyces graminofaciens* (75). The antibiotic is effective *in vivo.* It is soluble in organic solvents and has an empirical formula of $C_{28}H_{33}N_3O_7$.

Aureolic acid.—Aureolic acid (76, 77), an antibiotic active against gram-positive organisms and protozoa, is produced by an unidentified *Streptomyces* species. Toxicity data and mouse protection tests indicate that it is too toxic for chemotherapeutic use. The compound was obtained as the crystalline magnesium salt having an empirical formula of $(C_{56-60}H_{96-104}O_{29-31})_2Mg$.

Chartreusin.—Chartreusin (78) produced by *Streptomyces chartreusis* has an empirical formula of $C_{18}H_{18}O_8 \cdot 2H_2O$. The ultraviolet spectrum resembles a 1,2-naphthoquinone. It is active against gram-positive organisms and *Mycobacteria*, and chronic studies indicate an accumulative toxicity.

Broad Spectrum Antibiotics

Tetracycline.—The last review of the "Biochemistry of Antibiotics" in 1953 covered preliminary work which had led to the assignment of structures IX and X to chlortetracycline and oxytetracycline, respectively. Since that time, several articles have appeared describing in detail these and additional degradative studies. To adequately cover this material would require all the space allotted to this review. The work on oxytetracycline is covered in an excellent paper by Hochstein *et al.* (79) which gives in detail the reasoning and experimental work which led to the assignment of structure X. This paper also gives complete references to previous work. Another excellent paper is that of Stephens *et al.* (80) on the structure of aureomycin. This paper gives in detail the reasoning and experimental work, based on investigations with oxytetracycline, which confirmed the assignment of the structure IX to chlortetracycline. This paper likewise gives complete references to previous work.

The preparation of tetracycline, XI, by the catalytic removal of the chlorine from chlortetracycline was announced simultaneously by two groups (81, 82). The halogen was removed by reduction with hydrogen and palladium at atmospheric pressure and room temperature. The antibiotic is also produced by fermentation methods using a *Streptomyces* species (83). The *Antibiotics Annual* 1953–1954 contains a series of papers discussing the chemistry of tetracycline (84), *in vitro* studies with chlortetracycline, oxytetracycline, and tetracycline (85), a comparison of the three tetracyclines in experimental infections (86), the pharmacology of tetracycline (87), absorption and excretion studies, and clinical evaluation (88 to 92). In summary, tetracycline is more stable and soluble than chlortetracycline or oxytetracycline. Its biological activity and toxicity are very similar to that of chlortetracycline and oxytetracycline. Side reactions in patients may be less that that of the other two agents.

In addition to methods reported in the above references, Bird & Pugh have described a paper chromatographic method for the separation of tetracycline, chlortetracycline, and oxytetracycline (93); Sokolski *et al.* (94) have used *p*-dimethylaminobenzaldehyde as a spray for differentiating between chlortetracycline and oxytetracycline; and Perlman (95) has described a colorimetric method for the determination of chlortetracycline, carbomycin, erythromycin, and oxytetracycline. The latter method depends on the reaction of arsenomolybdate with acid hydrolysis products of the antibiotics. A colorimetric method based on metal-complex formation with chlortetracycline has been described (96). Chlortetracycline forms butanol soluble,

stable yellow complexes with certain divalent ions; calcium and cobalt were used. Albert (97) has studied the avidity of oxytetracycline and chlortetracycline for metallic cations. Results with the two antibiotics were the same. The order of preference for the various metals was much the same as with the common amino acids except for Fe^{++} and Fe^{+++} which are more strongly complexed by the antibiotics.

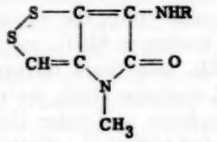
Chloramphenicol.—Most of the work on the chemistry of chloramphenicol during the past two years has been concerned with the preparation and testing of derivatives and analogues. Among the many compounds reported are the pyridine analogue (98), the *p*-benzyl, *p*-trimethylammonium, *p*-iodoxyl, and *p*-iodoso analogues (99), the *p*-hydroxy compound (100), the *p*-cyano, *p*-carbamoyl, *p*-ethoxycarbonyl, and *p*-thiazolyl analogues (101), methyl ethers (102), the *p*-trifluoromethyl analogue (103), side chain mercapto analogues and thiazolines (104), *p*-methylsulfonyl analogue (105), *p*-nitrophenyl and methyl ethers (106), the cyclic phosphoric acid ester (107), and the phosphite (108). The only compound having biological activity of the same order as that of chloramphenical was the *p*-methylsulfonyl analogue. Only the D-threo-isomer of chloramphenicol is active (109). The similarity of the structure of chloramphenicol to that of phenylserine has stimulated research on this compound and its stereoisomers as well as the stereoisomers of chloramphenicol (110 to 117). L(+)Threo-1-*p*-nitrophenyl-2-amino-1,3-propanediol has been used for the resolution of DL-acetyltryptophan (118).

Streptomycin.—Waksman has published two reviews covering the background, isolation, properties, and utilization of streptomycin (119, 120). Heuser *et al.* (121) have reported on the purification of streptomycin by use of salt formation with wetting agents of the sodium alkyl sulfate type. Measurements of the optical rotation of amorphous but chromatographically homogenous preparations of dodecabenzoyldihydrostreptomycin, the corresponding acetyl derivative, and of that of a crystalline methyl pentabenzoyldihydrostreptobiosaminide, together with other optical activity data, allow calculations to be made which demonstrate that the streptose-streptidine linkage in streptomycin is in all probability β-L and that the hexosamine-streptose linkage is in all probability α-L (122). It is pointed out that the hydroxyls in strepidine on carbon-4 and carbon-6, one of which is involved in the streptose linkage, are not sterically equivalent; this configurational point in the streptomycin molecule remains to be elucidated.

In an effort to clarify the role of glycine and other substrates in the production of increased streptomycin titers, an investigation of the extent of incorporation of glycine and acetate carbons into carbon dioxide, broth, cells, and streptomycin was undertaken (123). It was found that in a medium comprised of glucose, acetate, and glycine as the sole carbon sources, *Streptomyces griseus* utilized only the glucose carbons for the synthesis of streptomycin even though both acetate and glycine had to be present for efficient production of the antibiotic. More than half of the acetate and glycine car-

bon appears as carbon dioxide. The small amount of carbon incorporated from acetate and glycine was localized in the guanidine carbons of the molecule.

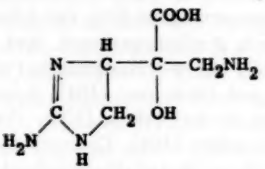
Thiolutin and aureothricin.—Thiolutin and aureothricin are similar yellow crystalline, sulfur-containing antibiotics isolated from different *Streptomyces* species (124, 125, 126). Both substances are active against a variety of fungi, ameboid parasites, gram-positive, gram-negative, and acid-fast bacteria. Thiolutin and aureothricin have been characterized as acetamido and propionamido derivatives, respectively, of a common hydrolysis



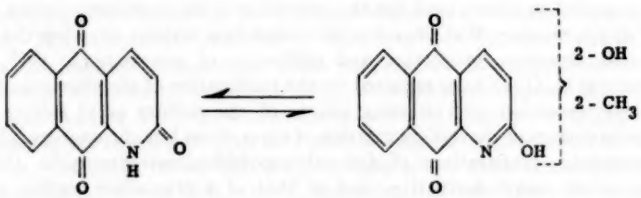
XII R = CH_3CO-

XIII R = H

XIV R = CH_3CH_2CO-



XV



XVI

product, $C_6H_6N_2OS_2$. Degradation studies and synthesis of degradation products (127, 128) have shown thiolutin, XII, to be the 3-acetamido derivative of 3-amino-5-methylpyrrolin-4-ono-(4, 3-d)-1,2-dithiole, XIII, and aureothricin, XIV, was shown to be the 3-propionamido derivative of XIII.

Thioaurin.—An antibiotic very similar in many of its properties to thiolutin and aureothricin is thioaurin (129, 130), $C_7H_6N_2O_2S_2$, which is active against a large variety of gram-positive and gram-negative bacteria and fungi. Its toxicity precludes its use in human therapy. Although thioaurin has the same empirical formula as the formyl derivative of the amine obtained by hydrolysis of thiolutin, it differs from this derivative in its melting point and absorption both in the infrared and ultraviolet regions (126).

Hygromycin.—This antibiotic is produced by *Streptomyces hydroscopicus*

and is active against both gram-positive and gram-negative organisms, *Actinomycetes*, *Mycobacteria*, and large viruses (131). Apparently it is nontoxic and has shown *in vivo* activity. It is a weakly acidic compound with a pK_a 8.9 (132). The noncrystalline compound is soluble in water and alcohol, has an $[\alpha]D^{25°}$-126° (C, 1, in water) and shows absorption at 272 mμ of $E_{1cm}^{1\%}=306$ and at 214 mμ of $E_{1cm}^{1\%}=416$. Hygromycin shows a positive reaction with Folin-Ciocalteu reagent but gives a negative ferric chloride test. It reduces both Benedict's and Fehling's solutions but fails to react in the anthrone, Molisch, or maltol tests. The antibiotic rapidly takes up eight moles of periodate, and the acetylated compound contains ten acetyl groups. The empirical formula is $C_{25}H_{39}NO_{12}$. Acid hydrolysis gives a crystalline base hydrochloride, $C_6H_{14}O_5NCl$. A second crystalline degradation product, $C_{10}H_{10}O_4$, was obtained by alkaline hydrolysis (132).

Streptothricin-Like Antibiotics

Streptothricin.—Carter *et al.* (133, 134) have reported on the preparation, properties, and hydrolysis products of streptothricin. Analyses of the crystalline helianthate and the amorphous hydrochloride indicate a tribasic substance with an empirical formula of $C_{20}H_{34}N_8O_9$. Titration data show three groups with pK_a^1 values of 7.1, 8.2, and 10.1. Benedict's, Fehling's, Tollen's, neutral permanganate, biuret, Pauly, ninhydrin, and Elson-Morgan tests were positive. On acid hydrolysis Van Slyke amino nitrogen increased to 75 per cent of the total nitrogen, but ninhydrin carbon dioxide increased only slightly. This showed that the antibiotic was not a so-called peptide antibiotic. On hydrolysis the antibiotic yields ammonia, carbon dioxide, and three ninhydrin-positive components. One of the hydrolysis products was identified as (+)-β,ε-diamino-*n*-caproic acid which was given the trivial name of β-lysine.

Streptolin.—Larson *et al.* (135) have shown that partially purified streptolin contains two components: streptolin A and streptolin B. Analyses of salts agree with an empirical formula of $C_{17}H_{31-33}N_5O_8$ or $C_{24}H_{45-47}M_7O_{11}$ for both compounds. Acid hydrolysis affords five major components which are: (*a*) a reducing fragment, (*b*) ammonia, (*c*) a basic substance possessing an empirical formula, $C_4H_{8-10}N_2O_3$, and (*d*) an isomer of lysine (136) which was synthesized and found to be identical with β-lysine isolated from streptothricin (135, 137) and viomycin (138).

Roseothricin.—Roseothricin is a streptothricin-like antibiotic obtained from *Streptomyces roseochromogenus* (139). Two products were isolated from acid hydrolyzates: (*a*) β-lysine (135, 137) and (*b*) 2-amino-4-(or 5)-(1-carboxy-1-hydroxy-2-amino)-ethyl-2-imidazoline, XV.

Roseomycin.—Roseomycin (140) and a closely related antibiotic (141) belong to the streptothricin group and are produced by the same organism as roseothricin.

Neomycin A.—Neomycin A was isolated from culture filtrates of *Streptomyces fradiae* following acid hydrolysis (142). The antibiotic had an em-

pirical formula of $C_{13}H_{26}N_4O_6$ and was shown to be identical with neamine which had been isolated as a degradation product of neomycin (143).

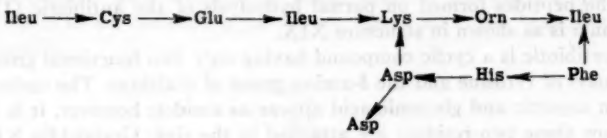
Xanthomycin.—The xanthomycins are characterized by the bright orange-yellow color of their solutions, a high activity toward gram-positive and gram-negative organisms and a high toxicity (144, 145). Xanthomycin A is a basic antibiotic with a molecular formula, $C_{22}H_{29-31}N_3O_7$, containing two salt forming groups, one methoxyl, one methylimide, and two terminal methyl groups. The compound has the properties of a benzoquinone having two centers of unsaturation, one of which is reversibly and the other irreversibly reduced.

Polypeptide Antibiotics

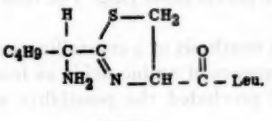
Actinomycins.—Waksman (146) has reviewed the actinomycins up to December, 1953. Although it was recognized then that the actinomycins, in common with other antibiotics containing a polypeptide moiety, consisted of a group of closely related compounds, more recent work has further emphasized the complexity of this group of substances. In a recent paper, Brockmann *et al.* (147), using countercurrent distribution and paper chromatography, have shown the presence of 30 actinomycins and have been able to characterize the seven obtained in crystalline form. Likewise, Vining & Waksman (148), using paper chromatographic methods, have shown the presence of additional compounds in actinomycins previously thought to be pure antibiotics. Brockmann *et al.* have published a series of papers on the purification and chemical nature of actinomycin (149 to 160). The compounds are all high melting, ranging from 232°C. to 246°C., and have specific rotations ranging from −309° to −349°. Molecular weight determinations by hydrogenation as well as by potentiometric titration in perchloric acid have given values of 1200 to 1350. The compounds have the same despeptide or chromophoric group. Brockmann agrees with Johnson *et al.* (161) that this chromophore has a molecular formula of $C_{15}H_{11}O_5N$ and adopted the azanthraquinone formula, XVI. A difference in the amino acid composition of the peptide portion of the molecules was found. Actinomycins I_2, I_1, C_1, X_1, and X_2, contain L-threonine, sarcosine, L-proline, D-valine and L-N-methylvaline. Actinomycin C contains D-allosioleucine in place of D-valine. In the case of actinomycin C_2 all six of the above amino acids are found. Sarlet (162) has also reported on the amino acid composition of an actinomycin AC. This antibiotic contains two molecules of L-proline, one of L-threonine, one of D-alloisoleucine, and four of N-methylated amino acids (sarcosine and N-methylvaline). Actinomycin AA has the same composition except for D-valine in place of D-alloisoleucine.

The actinomycins have probably been isolated many times by many different laboratories during the past fifteen years. Because of the extreme toxicity of the compounds very little was done with them until recently when it was reported that these antibiotics had a retarding effect on experimental cancers (163, 164).

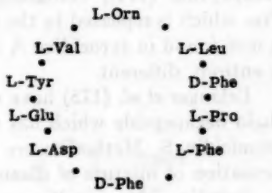
Bacitracin A.—Three groups of investigators have reported on structure studies on bacitracin A. An indication of the complexity of the molecule is the fact that somewhat different interpretations are placed on the data by each group. The reviewer has chosen to present the structure proposed by Craig *et al.* (165) and to point out where their interpretation differs from that of the others. By the partial hydrolysis of bacitracin 15 dinitrophenyl (DNP)



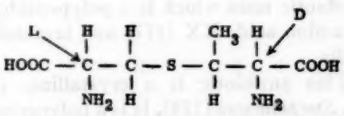
XVII



XVIII



XIX



XX

peptides were isolated by countercurrent distribution, paper chromatography, and paper electrophoresis. The molecule contains three isoleucine residues rather than two (166). An unknown band on the chromatogram (167) was identified as D-alloisoleucine in agreement with the results of Piez (168). The data and the finding that bacitracin as prepared retains one mole of acetic acid permit an over-all formula of $C_{66}N_{107}O_{19}N_{17}S$ to be written. This formula results from joining all the known fragments to form two rings. Craig's results could be rationalized by the following sequence, XVII, where→shows a C-N bond.

In the tri-DNP[2] derivative of bacitracin A the DNP groups were found attached to ornithine, histidine, and isoleucine. However, a low yield of DNP-isoleucine was obtained. Oxidation of the sulfur by performic acid improved the yield. On the basis of these results and absorption spectra studies a thiazoline ring structure, XVIII, is proposed.

The sequence reported here is in general agreement with those of Newton *et al.* (169, 170, 171). However, it does not agree with most of the sequences of Porath (172). Porath had previously proposed a thiazoline or thiazolidine ring structure in the molecule. It is possible that other cross linkages may exist and that rearrangments are taking place during breakdown.

Tyrocidine.—The structure of tyrocidine has been deduced by identification of the peptides formed on partial hydrolysis of the antibiotic (173). The formula is as shown in structure XIX.

The antibiotic is a cyclic compound having only two functional groups, the hydroxyl of tyrosine and the δ-amino group of ornithine. The carboxyl groups on aspartic and glutamic acid appear as amides; however, it is not known how these two residues are attached in the ring. Gramicidin S is a decapeptide (174), containing the sequence -L-Val-L-Orn-L-Leu-D-Phe-L-Pro- which is repeated in the cycle. It is of interest that this same sequence is now found in tyrocidine A as half of the polypeptide ring. The other half is entirely different.

Erlanger *et al.* (175) have reported the synthesis of a crystalline straight chain decapeptide which has the same sequence of amino acids as found in gramicidin S. Methods were used which precluded the possibility of the formation of mixture of diastereoisomers.

Subtilin.—A new sulfur containing amino acid was isolated from subtilin by Alderton (176). The structure is that of an α-amino-β-(2-amino-2-carboxyethyl-mecapto)-butyric acid, XX.

Nisin.—The antibiotic nisin which is a polypeptide resembling subtilin contains this same amino acid, XX (177) and lanthionine which is also a constituent of subtilin.

Amphomycin.—This antibiotic is a crystalline, potent antibacterial agent produced by a *Streptomyces* (178). It is a polypeptide with high surface activity. The antibiotic is an acidic compound with an isoelectric point of 3.5 to 3.6. It gives a positive biuret test and on acid hydrolysis gives ninhydrin positive materials separable by paper chromatography. The infrared spectrum is typical of a polypeptide and only end absorption is found in the ultraviolet region. Preliminary clinical trials indicate that at high doses amphomycin induces some hemolysis.

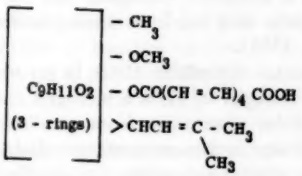
Fluvomycin.—Fluvomycin is a polypeptide antibiotic produced by *Bacillus sublitis.* It has *in vitro* activity against pathogenic bacteria and fungi but is inactive *in vivo* (179).

An antifungal agent (180) produced by a strain of *B. subtilis* is a polypeptide containing aspartic acid, glutamic acid, serine, threonine, alanine, tyrosine, norvaline, phenylalanine, and two unidentified substances. It is probably different from bacillomycin, mycosubtilin, and fungistatin which are also produced by *B. subtilis.* The substance is strongly hemolytic.

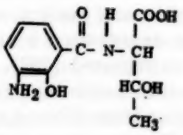
Brevin.—Brevin (181) is a polypeptide antibiotic resembling mycosubtilin. It contains aspartic acid, tyrosine, serine, glycine, and an unidentified basic substance.

Antifungal Compounds

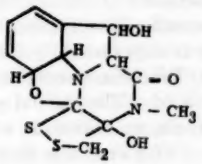
Fumagillin.—Fumagillin is an antibiotic with powerful amebicidal activity produced by an *Aspergillus fumigatus* (182, 183). Hydrolysis under mild alkaline conditions liberates decatetraenedioic acid, HOOC-(CH=CH)$_4$-COOH. On the basis of ozonolysis, hydrogenation, acid hydrolysis, alkali isomerization, and Kuhn-Roth determinations on the alcohol portion [$C_{16}H_{24}O_4$] of the molecule a partial structure, XXI, has been proposed for fumagillin.



XXI



XXII



XXIII

Mycomycin.—The structures of mycomycin and isomycomycin were reported in the last review (11). Celmer & Solomons have published two papers (184, 185), the first giving complete details on the proof of structure showing that the antibiotic is (-)-3,5,7,8-n-tridecatetraene-10,12-diynoic acid and the second the synthesis of two stereoisomers of methyl 3,5-*n*-tridecadienoates, 3(*trans*), 5(*cis*) and 3(*trans*), 5(*trans*). On the basis of the correlation of the properties of these model diene esters, mycomycin and isomycomycin were further characterized as possessing 3(*trans*), 5(*cis*) and 3(*trans*), 5(*trans*) stereoconfigurations, respectively.

Anchel (186) has isolated a crystalline antibiotic from the culture liquids of the Basidomycete, *Clitocybe diatretos*. The compound has an empirical formula of $C_8H_5NO_3$, and does not melt but explodes at 198°. The ultraviolet absorption spectrum indicated an ene-diyne structure. The reduction product of the antibiotic was identified as suberamic acid. Accordingly, the compound was assigned a structure of $H_2NOCCH{=}CHC{\equiv}C{-}C{\equiv}C{-}COOH$ or $H_2NOC{-}C{\equiv}C{-}C{\equiv}C{-}CH{=}CHCOOH$. Bu'Lock *et al.* (187) have isolated and synthesized two polyacetylenic antibiotics, one of which is identical with the second of the above two structures, the second being an

alcohol, $HOCH_2C{\equiv}C{-}C{\equiv}C{-}C{\equiv}C{-}CONH_2$, containing three triple bonds. Biformin I (188) is believed to be a 9-carbon glycol, containing two ethylenic and two acetylenic bonds in conjugation.

Antimycin.—Antimycin is produced by a *Streptomyces* and is of interest because it is capable, at very high dilutions, of blocking an essential step of the hydrogen transport system (189). The antibiotic has an empirical formula of $C_{28}H_{40}O_9N_2$ (190). Hydrolysis yields antimycic acid, $C_{11}H_{14}O_5N_2$, and a neutral fragment, $C_{16}H_{28}O_4$. The neutral fragment appears to be composed of two acids, one a ketoacid, $C_{11}H_{20}O_3$, and the other (+) methylethylacetic acid. Antimycic acid has been shown to be N-(3-aminosalicyloyl)-L-threonine, XXII, (191).

Other Antifungal Agents.—Candidin (192) is an antifungal agent produced by *Streptomyces veridoflavus*. It is a nitrogen containing acid which differs slightly in ultraviolet absorption from candicidin, ascosin, and trichomycin. Biological activity and countercurrent distribution also show it to be different from these antifungal agents. Candicidin (193) is a closely related compound produced by a *Streptomyces*. Ascosin, again closely related to candidin and candicidin, is active against yeast and filamentous fungi. The therapeutic dose approaches the toxic dose (194, 195). The antifungal activity is suppressed by fatty acids such as oleic, linoleic, and linolenic acids (196). Fradicin, prodigiosin, and fungicidin are also antagonized by these same acids. The partial purification of toximycin is reported (197). Trichomycin, an antibiotic with antifungal and antiprotozoal activities, is the subject of a recent review (198). The antifungal agent from *Monosporium bonorden* is probably mycophenolic acid (199, 200). The structures of three antibiotics, aurantiocladin, rubrogliocladin, and gliorosein, produced by a *Gliocaldium* species have been determined as 2,3-dimethoxy-5,6-dimethyl-*p*-benzoquinone the corresponding quinhydrone and dihydro derivative, respectively (201). Oligomycin, a new antifungal agent, $C_{25}H_{40-42}O_7$, is an unsaturated compound containing hydroxyl and carbonyl but no phenolic groups. The antibiotic is toxic (202). Studies on griseofulvin have continued (203). By replacing potassium chloride with potassium bromide in the medium a bromo-analogue was formed and isolated. Dechlorogriseofulvin was isolated from a culture of *Penicillium griseofulvum* (204). Fungisterol was shown to be $\Delta_{5,8,22}$ ergostatrienol (205). Squalene has *in vitro* activity against certain dermatophytes (206). *Escherichia coli* produces a substance which inhibits growth of fungi but has a marked toxicity (207). The study of the chemistry of gladiolic acid and related compounds has continued (208 to 211). Raistrick *et al.* (212) have reported on chemical studies with alternariol.

Miscellaneous Antibiotics

Azaserine.—The unique antibiotic azaserine has been characterized and synthesized. It is active against sarcoma 180 in mice, certain viruses, rickettsia, yeast, and some bacteria (213, 214, 215) and has had limited clinical

trial in patients with Hodgkin's disease, lymphatic leukemia, and acute leukemia (216). Azaserine was isolated from culture filtrates of a *Streptomyces* as light yellow-green crystals, showing as absorption maximum of $E_{1cm}^{1\%}$ 1140 at 250.5 mμ, and having the empirical formula, $C_6H_7N_3O_4$ (217). Degradative studies showed the compound to be O-diazoacetyl-L-serine, $N_2CHCOOCH_2CHNH_2COOH$ (218). Synthesis was accomplished by the selective diazotization of O-glycyl-D-serine (219, 220). An interesting sidelight is that diazouracil has been found to inhibit cell division of *E. coli* (221).

Sarkomycin.—Sarkomycin is an antibiotic produced by a *Streptomyces erythrochromogenes* (222). It has low antibacterial activity but is effective against Yoshida sarcoma in rats and Ehrlich carcinoma in mice. The compound is weakly acidic and extractable by organic solvents. It has not been obtained in pure form.

Anisomycin.—Anisomycin is a monobasic antibiotic isolated from two different species of *Streptomyces* and found to exhibit a high degree of *in vitro* activity against *Trichomonas vaginalis* and *Endamoeba histolytica* (223, 224, 225). It has very little antibacterial and antifungal activity. Titration data and analyses are in agreement with the formula, $C_{14}H_{19}NO_4$. The compound absorbs in the ultraviolet region with maxima at 224, 277, and 283 mμ.

Gliotoxin.—A crystalline antibiotic of molecular formula, $C_{13}H_{10}N_2O_6S_2$, was obtained together with gliotoxin from cultures of *Penicillium terlikowski* (226). The new substance was shown to be a monoacetate of gliotoxin. A new pentacyclic structure, XXIII, has been proposed for gliotoxin (227).

Actithiazic acid.—Actithiazic acid (see 11), (−)2-(5-carboxypentyl)-4-thiazolidione, has been synthesized (228) and a number of analogues prepared (229) and studied for *in vitro* antitubercular activity. Many of the esters and amides show greater antibacterial activity than the antibiotic itself. For maximum activity, a straight chain of five carbon atoms must separate the thiazolidone from the acid function. That actithiazic acid is an antimetabolite of biotin was confirmed (230).

Spicer (231) has reported that a *Micrococcus tetragenus* variant produces an antibiotic, trevison, which is active only against *Mycobacterium tuberculosis*. The crude substance is stable, nontoxic, and nonhemolytic. A crystalline antibiotic active against *M. tuberculosis* has been isolated (232, 233) from *Fusarium bostrycoides* and named bostrycoidin. It has an empirical formula of $C_{18}H_{14}O_7$, and forms an acetyl derivative. Its chemical properties indicate a naptho-1,4-quinone structure with two hydroxyl groups. The compound is probably similar to other *Fusarium* pigments. It is claimed to be nontoxic, but serum may interfere with its activity. Another substance showing *in vitro* activity against *Mycobacteria* is viridicatin, isolated from *Pencillium viridicatum* (234). The pure compound gives an analysis for $C_{15}H_{11}O_2N$. It is a phenolic polycyclic aromatic substance. Ethyl gallate was isolated from *Haematoxylan campechianum* (235, 236) and shown to have specific activity against *Mycobacteria*. It has a low toxicity and is active in

the presence of serum. The isolation of terreic acid from *Aspergillus terreus* was reported (237). The organism producing "ustin" has been shown to be *Aspergillus nidulans* (238). Since nidulin is a methyl ether of "ustin," it is proposed that the name for ustin be changed to nor-nidulin. Cardicin, produced by a *Nocardia*, has antimicrobial and antiviral properties but is toxic (239). A crude preparation of a substance active against tobacco mosaic virus was reported (240). It is produced by an *Actinomycete*. Rodocidin (241) is a toxic, unstable antibiotic produced by an undescribed *Streptomyces*. Elaiomycin is an antibiotic oil produced by a *Streptomycete* (242). It is active *in vitro* against *M. tuberculosis* but is ineffective against experimental tuberculosis.

Mode of Action of Antibiotics

The mode of action of antibiotics has been discussed in two recent reviews by Umbreit (2, 243) and was the subject of a published symposium by Wyss *et al.* (244). Those antibiotics which are useful in the treatment of systemic infections, i.e., those which can kill microorganisms without exhibiting toxicity to the host, have been studied intensively. A large portion of this effort has been spent in studying differences between sensitive and resistant strains, and this reviewer agrees with Umbreit (243) that this approach has been disappointing. Based on the large number of differences observed it is not considered to be of aid in pinpointing the biochemical reaction involved with any one of the antibiotics. Accordingly, the many references to such studies will not be covered. An excellent article by Eagle covers this subject (245).

The binding of penicillin by organisms and cell extracts of organisms has been studied by three groups of investigators. Eagle (246) finds a rough correlation between the amount of penicillin bound and concentrated by an organism and its sensitivity. However, resistant strains of an organism may bind more or less of the antibiotic than the parent strain (247). Since aqueous extracts of broken cells bind penicillin the same as the intact cells in all cases, permeability was not a factor. Penicillinase was also ruled out as a factor in these studies. Daniel & Johnson (248) agree with Eagle (246) that penicillin is irreversibly concentrated by the bacterial cell and aqueous extracts of the cells, and that the binding agent is nondialyzable and insoluble in lipoid solvents. In contrast, Copper *et al.* (249 to 252) find that the binding component is associated with a lipide fraction situated close to the cell wall, and they believe that penicillin brings about faulty function of the osmotic barrier. Such studies on binding of penicillin have the limitation that the molecule reacts with any grouping containing active hydrogen atoms making it difficult to identify the particular substance which is primarily responsible for inhibition of growth. As Eagle (247) points out, it may be less than 100 molecules of the 1600 to 3300 molecules bound per cell that are responsible for the inhibition. Eagle (253) has shown that penicillin is not bound by mammalian cells or their extracts, thereby eliminating the idea that the nontoxicity of penicillin to the host is attributable to permeability.

The isolation by Parke (254, 255, 256) of uridine-5′-pyrophosphate bound to an acetylamino-uronic acid from penicillin inhibited *S. aureus* has led to a study of the accumulation of this compound and related compounds under a variety of conditions. Parke (257) has found that other antibiotics and antibacterial agents bring about an increased formation of these compounds, and it may be that these changes are nonspecific and will be found under conditions such as aging. Strominger (258) has interpreted the results of experiments on the utilization of labeled uracil for the synthesis of nucleic acids to mean that penicillin was blocking nucleic acid synthesis. In a broad sense this is true, but it is still possible that the antibiotic is blocking a reaction such as the synthesis of a specific protein required for a step in the synthesis of a nucleotide or nucleic acid. Of particular interest concerning the problem of whether or not penicillin is involved in nucleic acid synthesis is an article by Cohen & Barner (259) on the unbalanced growth of an *E. coli* mutant requiring thymine. This organism in a complete medium except for thymine continues its cytoplasmic growth but does not synthesize deoxyribonucleic acid. At the same time there is extracellular production of uracil. In our own work (260) it was found that *E. coli* (ATCC 7923) inhibited by minimum amounts of penicillin gave results in many respects identical with those of Cohen & Barner. The organism accumulates extracellular uracil and continues to synthesize protein at a rate equivalent to that of the uninhibited culture. Protein synthesis continues up to an equivalent of about one cell division. This may indicate that in the case of penicillin, and perhaps other antibiotics, we are dealing with unbalanced growth which is not reversible.

In a paper presented at the New York Meeting of the American Chemical Society, Regna (53) pointed out the stereospecificity of the antibiotics. This is best illustrated by the work of Wisseman *et al.* (261) and Hahn *et al.* (262) with chloramphenicol. They showed that the D(−)threo isomer inhibited protein synthesis without affecting nucleic acid synthesis in *E. coli*, whereas the L(+)erythro isomer interferes with the formation of D(−)glutamyl polypeptide by *Bacillus subitilis*. They suggested an antipodal relationship between the steric configuration of the inhibiting drug and inhibition of protein synthesis. This conforms to the fact that only the D(−)threo isomer shows appreciable antibacterial activity. Gale & Folkes (263, 264) also found that chloramphenicol inhibits protein synthesis without affecting nucleic acid formation. Hopps *et al.* (265) interpret these observations, as well as the failure of the antibiotic to influence carbohydrate synthesis, as evidence in favor of a specific action of the antibiotic on protein syntheis. The inhibition of glucokinase formation in *E. coli* (266) and the inhibition of T_1 phage maturation can be explained on the same basis (267). Not enough is known concerning the stereospecificity of the other antibiotics to draw any conclusions. However, as a consequence of the synthesis of puromycin, there is an opportunity for studying the stereospecific action of carbohydrate substitution in this molecule.

The presence of an amino sugar in streptomycin, erythromycin, carbo-

mycin, picromycin, and puromycin is worthy of mention. Erythromycin and picromycin have similar antibacterial spectra, contain the same amino sugar, but differ considerably in their empirical formulas. Although it is necessary to recognize the possibility that the hexosamine containing pyrimidine nucleotides may not be involved in the action of the antibiotics, the presence of amino sugars in a number of antibiotics may indicate some relationship. A comparision of molecular models of 2-aminouronic acids with the antibiotics offers some interesting relationships. The ring in tetracycline carrying the dimethylamino group is very similar to a 2-aminouronic acid, with the dimethylamino group bearing the same relationship to the carboxamide group as the amino group has to the carboxyl group in a 2-aminohexuronic acid. The configuration of chloramphenicol is the same as that of glucosamine at carbon atoms two and three. Further correlations are possible, but until the functions of the hexosamine containing nucleotides are known the relationship between them and the antibiotics will remain speculative.

Newton (268, 269, 270) and Few (271, 272, 273) have published several articles on the mode of action of polymyxin. In summary, it was found that the antibiotic caused a leakage of pentose, phosphate, and materials which had an absorption of 260 mμ from the bacterial cell. This action could be reversed by magnesium. Rhodes *et al.* (274) observed that magnesium sulfate and magnesium ribonucleate reversed polymyxin activity against *Micrococcus pyogenes* var. arueus. Cohen *et al.* (275) showed that polymixin B caused marked inhibition of esterase activity of suspensions and extracts or microorganisms. Since the quaternary ammonium detergents gave similar inhibitory activity, they suggested a detergent-like structure for polymyxin-B.

The finding by Umbreit (276) that streptomycin inhibits the "oxalacetate-pyruvate" reaction at a concentration which also inhibits growth of *E. coli* has served to partially elucidate the action of this antibiotic. However, other strains of *E. coli* equally sensitive to streptomycin apparently do not possess the "oxalacetate-pyruvate" mechanism (277). Furthermore, the role of this seven-carbon phosphorylated tricarboxylic acid in metabolism is not known. Sevag *et al.* (278) and Bergman *et al.* (279) have reported the preparation of bacterial extracts which would reverse the effect of streptomycin. The relationship of the many observations, e.g., pantothenic acid metabolism (280) to the mode of action of streptomycin, is not clear. These references are well covered in Umbreit's reviews.

There have been several reports on metabolic changes brought about by the tetracyclines. Many of these changes can be explained on the basis of complexing of inorganic ions resulting in the uncoupling of oxidative-phosphorylation. A recent paper by Brody *et al.* (281), working with liver mitochondrial preparations, demonstrated uncoupling and inhibition of the oxidation of octanoate. The effect was greatest at low magnesium concentrations and negligible at high magnesium concentrations. They concluded that the effect was attributable to complexing of magnesium by the antibi-

otic. The concentrations are such as to indicate that this is not the mechanism by which the tetracyclines inhibit bacterial growth. The predominant effect found at growth inhibitory concentrations is an interference with protein synthesis (253).

Azaserine is reported to be a potent inhibitor against the incorporation of formate into purines (282), to interfere with tryptophan and phenylalanine metabolism (283), and to inhibit glucose fermentation and stimulate phosphate uptake in *Saccharomyces cerevisiae* with a parallel release of serine (284). These latter effects are reversed by leucine. Apparently the work on the mode of action of this antibiotic is developing much the same as with the older antibiotics in that many biochemical changes are being found which cannot be related to the inhibition of a specific chemical reaction.

This review of the mode of action of antibiotics covers less than 10 per cent of the articles which may contribute to the problem, and the reviewer admits that important contributions may have been omitted. However, it is hoped that these comments, taken together with those from other reviews, will at least point up some of the problems and be of help in reaching our objective of relating the structures of the antibiotics to their mode of action.

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CLINICAL APPLICATIONS OF BIOCHEMISTRY[1,2]

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The past 25 years have seen not only a vast acceleration in the application of biochemistry to medicine, but also a marked change in the manner of this application. During the years, 1915 to 1930, considerable advances took place in the development of quantitative semimicro or micro methods for the determination of blood, urinary, and fecal biochemical components. During this period the accumulation of data concerning the alteration of these components in various diseases helped build a stock of information that was of aid in the diagnosis and management of these diseases and, to some extent also, in the understanding of their underlying mechanisms. The development and perfection of such methods and the accumulation of such data have continued, but a new orientation has appeared, namely, the searching out of basic biochemical lesions in disease, very often on a cellular, enzymatic, or even molecular basis.

Although some clinicians think of biochemistry mainly in terms of its status before 1930 and of its aid chiefly as the immediate one provided by diagnostic procedures, an ever increasing number look to basic advances in biochemistry for illumination of their clinical problems. The newer role of biochemistry in medicine was cogently expressed in an editorial in Lancet (1) last year as follows:

> Biochemistry is the youngest and most active partner in the business of experimental medicine, and of all the sciences causes the greatest unease among clinicians. It is a new language, a new set of fundamental facts, a new mental approach to human physiology and pathology, and one largely created in the last twenty years.

The first part of the present review will consider those clinical applications of biochemistry which, as has been indicated, deal with the immediate diagnostic and management problems of the clinician and which should properly be termed "diagnostic biochemistry." The material in the second part will deal with biochemical mechanisms in disease and will be arranged in accordance with clinical entities. Obviously, because of the vast amount of work in these as in other fields, a selection is necessary, and omissions of the consideration of certain groups of diseases are inevitable.

[1] The literature considered in this review extends to and includes that of November, 1954.

[2] The following abbreviations are used: Ac-G for accelerator globulin; BAL for 2,3-dimercaptopropanol; EDTA for ethylenediaminetetraacetate; NCN for noncollagenous protein nitrogen; LSD for D-lysergic acid diethylamide tartrate.

DIAGNOSTIC BIOCHEMISTRY

Precision in the Clinical Laboratory

A growing literature notes with disquietude the considerable variability obtained by different clinical biochemical laboratories in the analysis of known prepared solutions, sera, or stabilized bloods. This problem was treated at some length by Wootton, Milne & King (2) last year, but because of its importance it deserves further consideration. In a survey of 59 laboratories, conducted by Belk & Sunderman (3) in 1947, about 50 to 70 per cent of the various determinations fell beyond the allowed experimental error. A similar degree of inter-laboratory variability was demonstrated by Wootton & King (4) in a survey of 21 hospitals with the use of a single specimen of whole blood preserved in acid citrate-glucose solution; for example, values for urea and uric acid ranged from 40 to 180 per cent of the mean value. Such variability is beyond that attributable to experimental error and to the possible use of methods of differing specificity in the different laboratories.

The reason for this variability merits study. In our present large hospitals and medical centers the cost of patient care has become a pressing problem (5). Biochemical analyses must of necessity be done in batches and on a "belt-line" basis, and the technicians who actually perform the determinations become a crucial factor in assuring accuracy and precision of the results. It is apparent that technicians should be well educated in the various branches of chemistry, but it is also important that they be properly motivated: for the performance of a specific technique day after day and, in some laboratories, month after month, is essentially a boring business, unless some appreciation of the meaning of the results is engendered.

The precision and accuracy of a clinical biochemical determination are also the end-result of a sequence of many steps, beginning often at the patient's vein. Some of these steps are seemingly trivial, but all are important. The supervising biochemist must exercise constant vigilance over each of these steps. He must guard his technicians against the laxness born of the error that the precision of the determinations need be merely as good as the biological variability of the components under analysis. The supervising biochemist, whether his primary training is medical or chemical, should be sufficiently acquainted with the clinical material so that any seeming discrepancy between the laboratory result and that expected from the patient's condition be immediately evaluated in terms of both laboratory procedures and clinical considerations.

Improvements in Analytical Methods

The flow of articles suggesting improvements in clinical biochemical methods continues steadily onward. Many of these suggestions are essentially minor ones. The merit of a proposed improvement should be evaluated in terms of whether, without sacrificing precision, the improvement shortens the time for performing the determination, lessens the cost, is more specific

for the component being analyzed, or eliminates hitherto unsuspected variability in the determination. A technical improvement may introduce a new value for a biochemical component into the literature, and subsequent clinical research may be necessary to determine whether there is an increased diagnostic advantage to the new value.

The application of flame photometry has made possible the rapid and precise determination of serum and urinary potassium and sodium and has permitted the finding and elucidation of many pathologic conditions which are characterized by abnormal regulation of these two ions. The need for a rapid flame photometric method for serum and urinary calcium determinations is already apparent in the study of the effect of various forms of treatment on osteolytic lesions in cancer (6). A number of such methods have been proposed (7, 8, 9), but because of low emission intensities, enhancement of intensity by sodium or potassium, depression by phosphate, or viscosity of serum, these methods required the usual substantial amounts of serum or plasma and laborious preparation of the sample for analysis through deproteinization (7), ashing (8, 9), or the addition of other ions. Denson (10) has proposed an adsorption column and subsequent elution to eliminate phosphates. Recently, Kingsley & Schaffert (11) and Winer & Kuhns (12) have found that organic solvents greatly increase flame photometric emission. As little as 0.02 to 0.1 ml. of serum or urine may be added directly to a mixture of acetone, acetic acid, and water or Sterox solution, which is then atomized in a Beckman DU spectrophotometer with flame and photomultiplier attachments, and readings are made at 422.7 mμ. Incidentally, the solvent also increases the emission intensity of sodium and potassium and thus permits the use of very small amounts of serum or urine in the determination of these ions. This procedure appears to offer greater hope for rapid calcium determinations than that based on the direct titration of serum with the chelating agent, disodium dihydrogen ethylenediaminetetraacetate (EDTA) (13), for the latter procedure suffers from an indistinct end-point, particularly in the presence of bilirubinemia. Spectrophotometric determination of the end-point, as recently proposed by Fales (14), circumvents this difficulty to some extent but, in the experience of this reviewer, an undesirable degree of arbitrariness is still involved in the graphical location of the end-point. Moreover, individualized titration-spectrophotometric determinations of the end-point are not sufficiently time-saving in comparison with the usual "belt-line" procedure demanded by the clinical laboratory.

In 1949 Gomori (15) introduced the principle of determining hydrolytic enzyme activity, e.g. phosphatase, by coupling liberated phenol with a diazo reagent to yield a colored compound. Seligman & Nachlas (16) utilized a similar principle in coupling β-naphthol liberated from β-naphthyl laurate by serum lipase or esterase with tetrazotized diorthoanisidine. More recently, Kaplan & Narahara (17) have further modified methods for phosphatase based on this principle by coupling phenol (liberated from disodium phenyl-phosphate) with Red B Salt. Fishman & Lerner (18), employing an earlier

observation that L-tartrate inhibits prostatic phosphatase, have devised a method for the determination of the prostatic acid phosphatase component of serum and have explored the possibility that such determinations may be of value in the diagnosis of localized or early carcinoma of the prostate.

There have been a number of other noteworthy improvements in standard methods or applications of analytic methods to the determinations of components in the blood, urine, body fluid, or feces. These include an adaptation of the color reaction between polysaccharides and anthrone to the determination of inulin and sucrose in plasma, urine, and other biological fluids (19, 20); the development of a photometric ninhydrin reaction by Moore & Stein (21), and the application of this reaction to the quantitative determination of plasma fibrinogen (22) and of gamma globulin in serum and cerebrospinal fluid (23, 24, 25). Wiese & Hansen (26) have published a method for unsaturated fatty acids of blood serum, based on spectral analysis of the alkali conjugated acids. A method for free choline in plasma which depends upon the solution of choline periodide in ethylene dichloride and the measurement of the optical density at 365 mμ has been developed by Appleton and co-workers (27). A number of methods have been proposed for cholesterol that are based on its interaction with ferric chloride to yield a stable purple colored solution (28). The conditions for the determination of urinary creatinine and creatine, which have been subject to some controversy and have led to reports of high urinary creatine excretion by the male, have been further investigated, and it has been shown that a pH of 2 to 2.5 is important in achieving maximal conversion of creatine to creatinine (29).

New Principles for Diagnostic Biochemical Determinations

The analysis of plasma or serum proteins in most diagnostic biochemical laboratories is still confined to determination of the total protein concentration and of the "albumin-globulin" ratio by means of the Howe technique. However, the necessity for a finer discrimination of the blood proteins has long been recognized. Studies of the electrophoretic patterns of the proteins of human plasma and serum in health and disease began to be done about 1939, and an impressive background of information now exists (30, 31). However, free electrophoretic analysis has been usually confined to selected cases because of the high cost of equipment, the necessity for specially trained personnel, and the time required for the analysis of specimens and computation of results.

During the past five years, methods have been developed for electrophoresis stabilized against convection by a supporting medium. This type of electrophoresis, known as "zone electrophoresis," has been applied to the analysis of serum proteins on filter paper by Kunkel & Tiselius (33), Levin & Oberholzer (34), Köiw and his associates (35), and a number of other groups of investigators. Paper electrophoresis has several advantages over free electrophoresis, namely, the use of very small amounts of serum (0.01 cc.), absence of a preliminary dialysis, less expensive apparatus, easy separation of

components after fixation, and the capacity for making several determinations of different sera on several filter paper strips. Automatic recording photometers for determining the relative concentrations of stained serum protein components are available. There is a great variety of design of paper electrophoresis apparatus, of the manner of making the run, and of the fixing and analysis of the protein components on the paper. These aspects have been reviewed by Fisher (31), Tiselius & Flodin (32), and most recently by Martin & Franglen (36).

The data already available indicate the practicability and usefulness of paper electrophoresis in the clinical biochemical laboratory. General protein disturbances in various pathologic conditions such as myeloma, the nephrotic syndrome, agammaglobulinemia, and portal and biliary cirrhosis, are readily revealed (37). The technique has also been used in the determination of other substances of clinical interest, such as urinary proteins, serum iron, and the various hemoglobins. McKay and co-workers (37) have found that results for the various serum components obtained by paper electrophoresis agree well with analysis by the Tiselius free electrophoresis method, except in the case of some abnormal sera, particularly those of high lipide content. Although the method has its limitations and continued investigation is necessary, there are already many indications that its use in the clinical biochemistry laboratory will be greatly extended.

During the past several years, a number of physical or chemical principles from other fields have been applied to analytical determinations of clinical significance. Thus, Shinowara (38) has outlined direct spectrophotometric methods for the measurement of hemoglobin and bilirubin in plasma. To circumvent the difficulties present in the colorimetric measurement of the iron-binding protein and capacity of serum, Feinstein and associates (39) have proposed a method whereby radioiron is added to serum and the serum proteins precipitated by ammonium sulfate; the iron-binding capacity is calculated by multiplying the specific activity of the standard solution by the difference between the radioiron added and the radioiron remaining in the filtrate. The application of paper chromatography to the detection of various components in plasma is growing rapidly; the determination of free amino acids by Gordon & Nardi (40) and of various barbiturate derivatives (41) may be cited as recent examples.

Methods for Newly Discovered Components in Blood, Urine, and Other Body Fluids

Diagnostic biochemistry depends almost entirely upon the analysis of components in readily obtainable materials such as blood, urine, cerebrospinal fluid, feces, and effusions of various kinds. The observation that a substance which hitherto has been found only in tissue, is also present in the blood or excreta immediately raises the problem of its quantitative determination and the possible clinical significance of such a determination.

The following recent advances in this field are of interest. The presence of various steroids in human blood has been investigated, and quantitative

methods have been proposed for the determination of 17-hydroxycorticosteroids (42), 21-hydroxy-20-ketosteroids (43), and neutral 17-ketosteroids in human plasma (44). Although the presence of adenosine pyrophosphates in human blood had been indicated a number of years ago, application of enzyme techniques has recently resulted in more precise and practicable determinations of the concentrations of these substances (45, 46). Fleisher (47, 48, 49) has launched a series of studies on the characterization of various peptidases in human blood, their possible tissue sources, and alteration of their activities in various diseases. Phosphohexose isomerase has been shown to be present in human serum (50). A method for its determination in serum has been devised and changes of its activity in patients with cancer have been studied (51, 52).

BIOCHEMICAL MECHANISMS IN DISEASE

Diseases of the Blood

Iron metabolism.—The excellent reviews by Laurell (53) and Granick (54) have summarized our recent knowledge of iron metabolism in health and disease. Absorption is increased in the anemias of iron deficiency, in pernicious anemia, and in refractory hypoplastic anemia. The concentration of transferrin, the protein that transports ferric ion in the serum, is expressed as total iron-binding capacity, and the difference between the total iron-binding capacity and the serum iron is designated as the latent iron-binding capacity. In late pregnancy and chronic iron deficiency, the concentration of serum iron is decreased and the latent iron-binding capacity is increased. In conditions characterized by increasing stores of iron (hemolytic, pernicious, aplastic, and myelophthisic anemias; hemochromatosis) the serum iron is increased and the latent iron-binding capacity is below normal. In acute and chronic infections, malignant tumors, and liver cirrhosis, the serum iron is decreased markedly, but the latent iron-binding capacity is decreased only slightly, if at all.

During the past year Bothwell and his associates (55) have shown, by use of isotopic techniques, increased iron absorption in idiopathic hemochromatosis and a slightly decreased absorption in malnutritional cytosiderosis. Iron absorption is decreased in steatorrheas with hypochromic anemia [Badenoch & Callender (56)]. Series of normal values for serum iron, copper, and free erythrocyte porphyrin have been determined in infants and children by Sturgeon (57). Employing a staining technique, Pratt & Johnson (58) observed increased iron stores in the marrow in the macrocytic anemias and in normocytic anemias not attributable to acute bleeding, and decreased iron stores in patients with chronic bleeding and in infants with nutritional anemias.

Blood clotting mechanisms.—Recent investigations have evoked an increasing array of factors that are involved in the activation or acceleration of the conversion of prothrombin to thrombin, in the formation of these activators, in the role thrombin plays in converting fibrinogen to fibrin, and in

the destruction of the blood clot. These factors are chemically ill-defined at the present time and have often been designated in different ways by different investigators. Stefanini (59) and Milstone (60) have summarized current concepts of the mechanisms for blood clotting.

During the past year Lewis & Ware (61) submitted additional evidence that there is only one factor that accelerates the conversion of prothrombin in human plasma; plasma accelerator globulin (Ac-G) exists in human plasma in a relatively inert form and is mostly but not entirely converted to the much more active serum Ac-G by very small amounts of thrombin. Rosenthal (62) has extended his studies on the various plasma thromboplastin factor deficiencies that may be responsible for hemophilia and hemophilia-like diseases and has studied the frequency of anti-hemophilic globulin (AHG) deficiency, plasma thromboplastin antecedent (PTA) deficiency, and the plasma thromboplastin component (PTC) deficiency in a group of 33 patients. Ratnoff & Potts (63) evaluated more precisely the accelerating effects of calcium and other cations on the conversion of fibrinogen to fibrin by thrombin at constant ionic strength. More recently Ratnoff (64) has found normal plasma to contain a heat-labile nondialyzable component which accelerates the coagulation of fibrinogen by thrombin. Sherry (65) has shown that the fibrinolytic activity of purified human plasmin is of the same order as other proteolytic enzymes and has studied the activating effect of streptokinase.

Variants of hemoglobin.—An impressive illustration of understanding disease in terms of underlying biochemical lesions is found in the group of the hereditary anemias of the sickle-cell, thalassemia and related and inter-connected groups. The reviews by Drabkin (66), White (67), and most recently by White & Beaven (68) have dealt with the structure of the hemoglobins in these anemias and with physico-chemical methods for recognizing differences between them. To the five variants of hemoglobin (hemoglobins A, F, S, C, and D) Itano, Bergren & Sturgeon (69) have recently added a sixth, hemoglobin E, found in the red cells of a child with an atypical anemia and having an electrophoretic mobility greater than that of Hb-A but slightly less than that of Hb-S.

Using paper electrophoresis of lysed red cells to distinguish the various hemoglobins, Smith & Conley (70) have surveyed their distribution and incidence in the patients of a random clinic population; they found no S, C, or D hemoglobin in any of 500 white persons, and hemoglobins S and C in 8.4 and 2 per cent, respectively, of 500 negroes. One person had S-C hemoglobin combination or sickle-cell hemoglobin disease, a condition which appears to be characterized by relatively mild hemolytic anemia, rarity of violent crises, persistence of splenomegaly into adult life, and the presence of large numbers of target cells in the blood smear. Pure hemoglobin-C disease has been reported by Spaet, Alway & Ward (71) and more recently by Singer and his associates (72). A new syndrome, hemoglobin-C-thalassemia disease, has been described by two groups of workers (73, 74). The interre-

lationship between hemoglobin structure and the anemias is an extremely active field of investigation at present, but limitation of space precludes further discussion.

Diseases of the Heart and Cardiovascular System

Blood lipides and atherosclerosis.—The thesis of Gofman and associates (75, 76, 77) that certain of the serum lipoproteins, characterized by their flotation rates, are specifically correlated with the occurrence of human atherosclerosis has been subjected to criticism, chiefly by Keys (78, 79, 80). The latter has submitted statistical analyses that total serum cholesterol provides as good, if not a better, discrimination between coronary and noncoronary populations than do lipoprotein measurements and that both have a low order of diagnostic or prognostic value. Within the past year Gofman and co-workers (81) reviewed their extensive data and have shown that the concentrations of the components, S_f0 to 12 and S_f12 to 400, were significantly higher in males of 40 to 49 and 50 to 59 years of age with coronary artery disease than in noncoronary men of these age groups, but that there was no difference in the 60 to 69 year-old groups. An expression which is a function of the two lipoprotein components and the diastolic blood pressure has been proposed by Gofman *et al.* (81) as a means of estimating the relative risks of development of human coronary heart disease. Moreover, Gofman and co-workers have denied the validity of Keys' conclusion concerning the use of total serum cholesterol estimations, pointing out that the latter's statistical analyses were not based on determinations of cholesterol and lipoprotein in aliquots of the same blood.

Barr (82) has recently reviewed the results of the application of the chemical fractionation technique of Cohn (83) to the problem of atherosclerosis. Barr studied the distribution of the concentrations of total cholesterol, phospholipide, and the fraction of cholesterol in the α- and β-lipoproteins in the serum of normal and atherosclerotic dogs and rabbits, in the serum of normal infants, of males and females of various ages, of patients with various lipide disorders and of survivors of myocardial infarction. Although Barr (82) has made no rigid statistical evaluation of his data, the following comparisons are available: 61 per cent of survivors of myocardial infarction had 15 per cent or less of total cholesterol in the α-lipoprotein component, as contrasted with 19 per cent of a control group of males aged from 45 to 65, 10 per cent of a control group of women of this age range, and 8 per cent of a control group of men 18 to 35 years of age.

Estrogen administration to survivors of myocardial infarction caused a decrease in the total plasma cholesterol, an increase in the cholesterol fraction of α-lipoprotein, and a decrease in the cholesterol/phospholipide ratio (82, 84). Administration of methyl testosterone decreases the cholesterol fraction of α-lipoproteins (82). This decrease may perhaps be related to an androgen effect in atherosclerosis, for Wuest and co-workers (85) found by histologic study of necropsy material that the degree of coronary sclerosis in bilaterally

oophorectomized women was greater than in control women but less than in control men. Blumgart and co-workers (86) were unable to detect any abnormal degree of atheromatosis or atherosclerosis in eight patients who had been maintained at levels of hypothyroidism of about minus 20 per cent for one to 13 years following thyroidectomy and who had shown an average increase in the blood cholesterol of 125 mg. per 100 cc. above the pretreatment level.

The relation of the nature of the diet to blood lipide fractions and to the occurrence of atherosclerosis has naturally commanded much attention. Keys and his co-workers (87) recently studied a group of clinically healthy men in Naples, 20 to 55 years of age, who were matched with a group of Minnesotans in size, weight, obesity, and total caloric intake, but whose diet contained 20 per cent of the total calories in the form of fat as compared to 42 per cent in that of the Minnesota group. The rise in the average serum cholesterol concentration from age 20 to the early thirties was only about 3 mg. per 100 cc. in both the Neapolitan and the Minnesota groups. Beyond this age the serum cholesterol in the Neapolitans showed no further increase. However, in the Minnesota group the serum cholesterol concentration continued to rise until at age 50 it was greater than that in the Neapolitan group by about 30 mg. per 100 cc. Similar findings were obtained by Walker & Arvidsson (88) in a group of South African Bantus who had been subsisting on a low fat but calorically adequate diet. Higginson & Pepler (89) found a lower frequency of severe atherosclerosis and coronary artery disease at post mortem among Bantu patients than among patients from American and Danish hospitals. That the total fat in the diet and not the cholesterol is related to the plasma levels of cholesterol is evident from the study of Mayer and his associates (90) who placed five normal adults successively on various diets. A low-fat low-cholesterol diet led to significant decreases in the plasma cholesterol levels, and the addition of large amounts of cholesterol as egg yolk did not cause any change. However, an increase in the proportion of dietary fat was associated with a significant increase in plasma cholesterol levels.

Heart failure.—The metabolic adjustments to physical activity have been compared by Newman (91) in normal persons and in patients with congestive heart failure; the patients with cardiac insufficiency showed a greater decrease in sodium and chloride excretion and a slower return to normal excretion. Although it has generally been found that the plasma volume is increased in heart failure, Eisenberg (92), employing P^{32}- or Cr^{51}-tagged red cells, was not able to obtain evidence of any increase in total blood volume, plasma volume, or red cell volume. Schreiber and co-workers (93) considered the possibility that this discrepancy might be attributable to a decrease of the ratio, total body relative cell volume/venous hematocrit value, in heart failure. Upon performing simultaneous red cell and plasma volume determinations, they found that the mean values for red blood cell volumes and plasma volumes were elevated above those of control subjects and fell with

compensation. In high-output heart failure attributable to beri-beri, pronounced cellular uptake of potassium occurred during recovery (94). Talso *et al.* (95) compared the composition of muscle tissue taken from patients in heart failure and after compensation. The tissue in heart failure was characterized by increases in both extra- and intracellular water, increased sodium and chloride content, and decrease in intracellular potassium.

A number of reports deal with other biochemical aspects of heart failure. Kleeberg & Gitelson (96) have reaffirmed previous reports in the literature that blood pyruvic acid is elevated in patients with congestive heart failure. Sutton and co-workers (97) found that whole blood creatine was elevated in heart failure, that the extent of elevation bore a relation to the degree of decompensation, and that blood creatine decreased on recovery. Conversely, the concentration of creatine in skeletal muscle, as determined by analysis of biopsy material, was low in heart failure and rose when the patient was compensated.

Metabolism of the human heart in failure.—Metabolic studies of cardiac tissue have always been of considerable interest, but most of these have been performed on animals and under essentially *in vitro* conditions. The main features of studies of this type on the failing heart were reviewed by Olson & Schwartz (98) in 1951. Bing and his co-workers (99, 100) have recently undertaken a series of investigations *in vivo* which have been facilitated by the development of a technique for catheterizing the coronary sinus. The extraction of glucose and lactate by the myocardium was elevated both in low and high output failure. The glucose and lactate oxygen ratios or, in other words, the fractions of the total oxygen extraction, calculated as a result of the combustion of glucose and lactate, were also elevated. The energy conversion factors for these two substances, that is, the values obtained by dividing the work of the left ventricle by the glucose or lactate oxygen ratio, were lowered. The results indicate that the hyper- and hypokinetic heart in failure does not convert energy derived from the aerobic breakdown of glucose and lactate into mechanical work as readily as the normal heart. Bing and his associates have also studied the metabolism of fat, ketones, and amino acids by the normal heart *in vivo* (100).

Diseases of the Kidney and Prostate

Nephrosis.—The biochemical features of the nephrotic syndrome have recently been the subject of renewed interest by a Rockefeller Institute-New York Hospital group (101 to 104). Chinard and associates (101) found that the glomerular permeability to albumin is increased considerably at induced normal plasma levels of this protein. Infusion of albumin into the nephrotic patient caused an increase in plasma volume and indicated the presence of a previously decreased plasma volume (102). Earlier evidence in the literature indicated that the accumulation of edema in the nephrotic patient was attributable to the renal tubular reabsorption of filtered sodium, chloride, and water in excess of that necessary for the maintenance of a normal ex-

tracellular fluid volume. This increase in tubular reabsorption could be ascribed either to a direct action of the tubules or to a decrease in the rate of glomerular filtration (glomerulo-tubular imbalance). To test these alternatives, Eder and associates (103) measured various renal functions before, during, and after periods of spontaneous or infused albumin-induced diuresis, and their results indicated the following mechanisms. Increased glomerular permeability to albumin and the resultant albuminuria lead to hypoalbuminemia and to a shift of water and various solutes from the plasma to the interstitial space. The resultant decrease in the plasma and blood volume leads to some degree of circulatory inadequacy which in turn may cause renal ischemia, decreased glomerular filtration rates, a glomerulo-tubular imbalance, an excessive tubular reabsorption of sodium, chloride, and water, and ultimately edema. Lauson and co-workers (104) have presented evidence indicating that the favorable therapeutic effect of ACTH in children with nephrosis consists in a correction of the increased glomerular permeability to albumin and other proteins.

Of interest also are the findings of Cartwright, Gubler & Wintrobe (105) that the concentrations of serum copper and iron are decreased in most nephrotics. They have suggested that these decreases may be associated with the urinary loss of the proteins, ceruloplasmin and transferrin. Rosenman, Friedman & Byers (106) observed that increases in the concentration of serum cholic acid were in parallel with increases in the concentration of serum cholesterol in nephrotic patients, a parallelism also observed in rats rendered nephrotic by injection of rabbit anti-rat kidney serum.

Derangements in acid-base regulatory mechanism of the kidney.—A number of diseases characterized by acid-base disturbances can be understood in terms of the regulatory functions of the kidney, as formulated in publications from the laboratories of Pitts (107) and others (108). Carbon dioxide is hydrated in the tubular cells to carbonic acid, a reaction that is catalyzed by carbonic anhydrase; the carbonic acid, as a dissociable acid, yields hydrogen ions which pass through the tubular cell wall and are excreted as such (titratable acid), or combine with NH_3^+ to form NH_4^+, or exchange with Na^+. Schreiner and co-workers (109) recently reported two cases of renal hyperchloremic acidosis, and Wyngaarden and associates (110) reported a patient with malignant nephrosclerosis who had alkalosis, hypochloremia, hypokalemia, and hyponatremia. Roberts and co-workers (111) have studied the renal compensations involved in postoperative alkalotic patients. The inulin (extracellular) space and the total extracellular content of sodium, bicarbonate, and chloride, but not of potassium, were increased. Metabolic studies revealed large losses of potassium. In contrast to the situation in the normal person, bicarbonate was absent from the urine at plasma bicarbonate concentrations greater than 27 to 29 m.eq. per l. These findings were interpreted to indicate that tissue potassium depletion results in a decrease of intracellular pH, including that of the renal tubular cells, and that these decreases in renal tubular intracellular potassium and pH potentiate the renal

tubular reabsorption of bicarbonate, with a resultant plasma alkalosis and the excretion of an acid urine.

Carcinoma of the prostate.—The association of elevated serum acid phosphatase activity with cancer of the prostate continues to be of interest. The subject of acid phosphatases has been comprehensively reviewed by Walker and associates (112). In applying their test for estimating serum acid phosphatase of prostatic origin (17), Fishman *et al.* (113) found that all of seven patients with metastatic carcinoma of the prostate gave total and "prostatic" serum acid phosphatase values that were elevated above the normal range. Of five patients with localized prostatic carcinoma and normal total acid phosphatase, four had elevated values for the "prostatic" acid phosphatase component.

Baker and co-workers (114, 115) observed that serum aldolase activity was increased in 12 of 16 patients with proved, untreated, advanced cancer of the prostate. Hormonal or diethylstilbestrol therapy, which produced clinical remission, was usually accompanied by a decrease of this enzyme activity to normal levels. In connection with the problem of correlating serum enzyme activity and clinical status it is well to note that enzymes like aldolase, which are involved in essential metabolic sequences, are likely to be present in many tissues and that any effect on the integrity of these tissues may cause the release of enzyme into the blood. For example, Schapira and co-workers (116) have noted greatly elevated levels of serum aldolase in patients with various myopathies.

Diseases of the Liver

Ascites and edema in cirrhosis.—Several recent studies (117, 118, 119) have considered further the role of oncotic pressure and derangements of electrolyte and water metabolism in the production of ascites and edema. However, the thesis that antidiuretic substances also are a factor has been severely challenged. Van Dyke and his associates (120) failed to confirm the presence of significant antidiuretic activity in the urine of cirrhotic patients when the dog was used for assay. White, Rubin, & Leiter (121) reported that the rate of inactivation of intravenously administered beta-hypophamine (Pitressin) was normal in patients with hepatic cirrhosis; they could obtain no evidence of overproduction. Miller & Townsend (122) observed no difference between normal and cirrhotic human liver in the inactivation, *in vitro*, of beta-hypophamine. Employing the rat as an assay animal, Stein and associates (123) found that the elevation in antidiuretic activity of the plasma of cirrhotics with edema was about 28 per cent above normal but was not significantly different from that in hypertension or in patients with various miscellaneous diseases.

Blood ammonia and electrolytes in hepatic coma.—Within the past few years the question has arisen whether "hepatic coma," a neurologic manifestation of severe and frequently preterminal hepatic impairment, is related

to a derangement of nitrogen metabolism and the presence of high concentrations of ammonia in the blood and tissues. Phillips *et al.* (124) reported that the characteristic mental disturbances, flapping tremor, and distinctive encephalographic changes in impending coma could be reproduced in patients with advanced cirrhosis by the administration of ammonium potassium cation exchange resin, ammonium chloride, di-ammonium citrate, or urea, or by the feeding of increased amounts of protein. McDermott & Adams (125) reported similar results could be produced in a patient with a surgical Eck fistula when urea or ammonium chloride was given by mouth.

There does not, however, appear to be any consistent relationship between the levels of blood ammonia and the extent of clinical manifestations of hepatic coma. Although patients with actual or impending coma generally have elevated levels of blood ammonia, there are some who do not and, conversely, some cirrhotics show high levels without having any symptoms of coma (126, 127). The possibility that other biochemical factors may be involved in hepatic coma has been explored by Schwartz and co-workers (126) who failed to elicit any relationship between hyponatremia or hypokalemia and the neurologic status. Artman & Wise (128) found hypokalemia in 25 out of 30 cases of liver cirrhosis in hepatocellular failure, but in many of these the hypokalemia was associated with preceding anorexia, nausea, diarrhea, and vomiting and hence was not necessarily related to the characteristic neurologic signs of hepatic coma. Walshe (129) reported that patients in hepatic coma may show increased concentrations of plasma glutamine, but Seegmiller *et al.* (130) failed to obtain any such correlation, either serially in individual patients or by comparison of groups.

Glycogen storage disease.—In an analysis of several cases of this disease, the Coris and their associates (131, 132, 133) distinguished four types on the basis of the glycogen structure. In the first type which included 10 cases, characterized by deposits of glycogen in the liver and kidney (von Gierke's disease), and in the second type which consisted of one case of generalized glycogenosis, the glycogen structure was normal, that is, about 7 to 8 per cent of the bonds were 1,6-bonds and about 30 per cent of the glycogen was degraded by phosphorylase. In another case with abnormal storage in liver and muscle, the glycogen was abnormal; it had about 11 to 13 per cent of 1,6-bonds and was degraded to a slighter degree, namely about 2 to 12 per cent, by phosphorylase. In another patient, these features were reversed, namely, the glycogen had a low percentage of 1,6-bonds and was degraded to the extent of 50 per cent by phosphorylase.

Glycogen is converted to glucose through the following sequence of enzymes: phosphorylase, amylo-1,6-glucosidase (debrancher), phosphoglucomutase, and a specific glucose-6-phosphatase. The reverse sequence involves, in addition to phosphorylation and the other reactions, that mediated by the brancher, amylo-(1,4→1,6)-transglucosidase. The Coris (131, 132, 133) found glucose-6-phosphatase of liver homogenates to be greatly reduced in

six cases with glycogen disease of the liver, of which three were fatal. In five other cases with relatively mild symptoms, the glucose-6-phosphatase was either moderately depressed as in other liver diseases or within the normal range. Harris & Olmo (134) have confirmed these findings in two cases of liver glycogen storage disease.

The liver glucose-6-phosphatase activity was normal in the single case of generalized glycogenosis (131). Therefore, the storage of glycogen in the liver in this case could not be explained either by abnormality of glycogen structure or by absence of the specific glucose-6-phosphatase. Moreover, since glucose-6-phosphatase is not normally present in skeletal or heart muscle, its absence cannot be related to the presence of the generalized storage in this variety of glycogen disease. In the case with a high percentage of 1,6-bonds and a low degradation by phosphorylase, the enzyme defect would appear to be a deficiency of amylo-1,6-glucosidase (debrancher), and in the case characterized by low content of 1,6-bonds and high phosphorylase degradation, it may be deduced that there is a deficiency of the branching enzyme, amylo-(1,4→1,6)-transglucosidase.

Copper and amino acid metabolism in hepatolenticular degeneration (Wilson's disease.)—This rare but biochemically interesting disease was reviewed in detail by Wootton, Milne & King (2) in last year's *Annual Review of Biochemistry*. It is characterized by aminoaciduria, an increased storage of copper in the liver, basal ganglia, and the Kayser-Fleischer ring in the cornea, a decreased concentration of serum copper and of the copper-containing serum protein ceruloplasmin, and an increased urinary excretion of copper (2, 135). These findings have been explained by assuming an increased intestinal absorption and tissue accumulation of copper (a derangement of copper metabolism such that a minimal portion is excreted in the feces), renal tubular damage, chelation of amino acids with copper, and excessive urinary excretion of the chelated amino acids (135).

Some aspects of this formulation have been confirmed by Zimdahl and associates (136) who reported a reduced fecal excretion of copper and a substantial positive balance of copper despite the high urinary excretion. BAL (2,3-dimercaptopropanol), ACTH, EDTA,[3] and an ion exchange resin increased the excretion of copper and decreased the positive balance or sent the patient into negative balance. With BAL and EDTA the fraction excreted through the urine increased (137). Bearn & Kunkel (138) have reported that the mean serum copper concentrations were 108 μg. per 100 cc. in a group of 11 normal persons, 171 μg. per 100 cc. in a group of 20 patients with cirrhosis, and 60 μg. per 100 cc. in 17 patients with Wilson's disease. The urinary copper excretions in μg. per 24 hr. were, 48 in the normal persons, 157 in the cirrhotic patients, and 703 in those with Wilson's disease. These findings have in general been recently confirmed by Cartwright *et al.* (139) who have also reported an elevated concentration of copper in all tissues except the heart, skeletal muscle, lung, and erythrocytes.

The aminoaciduria has been further studied by Stein, Bearn & Moore (140); the amounts and the qualitative distribution of amino acids were found to depend on the state of the disease and on the level and composition of the protein intake. The increase of urinary amino acid excretion in response to a stated increase of protein was much greater in Wilson's disease than in normals. Increasing the intake of copper did not produce an increase in the urinary excretion of amino acids by patients with Wilson's disease, but the increased aminoaciduria as a result of high intake of protein or administration of cortisone was accompanied by an increase in copper excretion. The converse situation did not however hold; increases in urinary copper attributable to administration of BAL or EDTA were not accompanied by an increased amino acid excretion (138).

Diseases of Metabolism

Diabetes.—In a detailed and critical review Stadie (141) has summarized current concepts concerning the possible locations of metabolic blocks in insulin deficiency or in the presence of an excess of contra-insulin principles. These are the transfer of glucose across cell membranes or interfaces, phosphorylation of glucose to glucose-6-phosphate through the intermediation of glucohexokinase, regeneration of adenosinetriphosphate (ATP), and oxidative reactions involving the Krebs cycle. The thesis proposed by Levine *et al.* (142, 143) that insulin promotes the rate of transfer of glucose and structurally similar monosaccharides across cell barriers has received confirmation from other laboratories (144, 145, 146). Demis & Rothstein (147) have shown that insulin stimulates the uptake of glucose, fructose, and galactose by excised rat diaphragm under aerobic but not anaerobic conditions. Fisher & Lindsay (148) have demonstrated that insulin increases the rate of transfer of galactose through the cell membrane in a perfused rat heart preparation. With respect to the possibility that glucohexokinase activity may be affected by insulin, Auld (149) has recently reported that this enzyme activity is the same in homogenates of skeletal muscle from diabetic human subjects as from normal persons. Moreover, the addition of insulin *in vitro* did not alter the hexokinase activity in either group.

The results of Chaikoff (150) that liver slices of the alloxan-diabetic rat could oxidize fructose but not glucose appears to have stimulated investigations on the utilization of these hexoses in normal and diabetic humans. After intravenous injection, fructose disappears much more rapidly than glucose in both normals and diabetics; whereas the utilization of glucose is greatly impaired in diabetes that of fructose is only slightly retarded (151, 152, 153).

Pancreatitis and tumors of the pancreas.—The blood coagulation factors have been found to be altered in these conditions, presumably as the result of elevated blood trypsin concentrations (154, 155). On the basis of a study of 192 patients with and without proved pancreatic disease and from an assess-

ment of the earlier literature, Dreiling & Richman (156) have concluded that provocative blood enzyme tests have no value in the diagnosis of pancreatic disorders.

Pancreatic cystic fibrosis.—Di Sant'Agnese and co-workers (157, 158, 159) have found that the sweat from patients with this disease contains abnormally high concentrations of sodium and chloride and moderately elevated concentrations of potassium. These derangements are not attributable to impairment of renal or adrenal function, but apparently to a disturbance in the sweat glands. In hot weather this disturbance may lead to considerable decreases in the sodium and chloride concentrations of the extracellular fluid and thus account for the high incidence of heat casualties seen in children with pancreatic cystic fibrosis. According to di Sant'Agnese (159), these observations, together with preliminary findings that the volume of saliva and the concentrations therein of sodium and chloride are elevated, indicate that fibrocystic disease of the pancreas may represent a multiple disturbance of glandular structures.

Human nutrition.—Until recently the role of vitamin B_6 in human nutrition was equivocal (160). During 1952 and 1953 analysis of several episodes of unexplained epileptiform convulsions in infants about 8 to 16 weeks of age who were being artificially fed with a particular liquid proprietary preparation led to the surmise that this preparation was producing a deficiency in pyridoxine (161, 162). Intravenous or intramuscular injections of pyridoxine corrected such convulsions (163) as well as those induced by a prolonged pyridoxine-free diet [Snyderman and co-workers (164)].

The amount of pyridoxine necessary to prevent the syndrome of epileptiform convulsions has not been determined and may vary greatly; the level in the liquid proprietary preparation, the feeding of which originally led to the syndrome, was not much less than that present in human milk (161). Hunt and associates (165) have reported the case of an infant who had repeated bouts of convulsions during the first year of life on an adequate milk formula supplemented by 300 μg. of pyridoxine per day. These convulsions were stopped immediately by injections of 6 mg. pyridoxine and were controlled by daily oral doses of 2 mg. One of the effects of vitamin B_6 deficiency is a derangement of the normal sequence of reactions involved in the conversion of tryptophan to nicotinic acid, and xanthurenic acid is excreted in the urine in large amounts. Vilter and co-workers (166) obtained several-fold the normal excretion of xanthurenic acid after administration of desoxypyridoxine to adults.

In view of the general role of protein and essential amino acids in human nutrition, it is conceivable that aminoaciduria may contribute to malnutrition. Jonxis & Huisman (167, 168) have reported that infants and children suffering from deficiency of vitamin C or vitamin D showed an excessive urinary excretion of amino acids. The increase in amino acid excretion in rickets consisted, both in free and bound form, of lysine, histidine, glycine

plus alanine, glutamic acid, and threonine plus serine (167). Jonxis & Huisman (168) reported two infants with scurvy whose daily excretions of urinary α-amino nitrogen were two- to three-fold those of controls; the increased excretion consisted of serine, glycine, tyrosine, histidine, α-amino isobutyric acid and of the essential amino acids, threonine and lysine.

Treatment of scorbutic children with vitamin C reduced the degree of aminoaciduria to normal levels (168). Jonxis (167) has, however, reported that treatment of rickets with vitamin D did not reduce the aminoaciduria completely to normal levels and that adults who had had rickets in childhood continued to have some aminoaciduria. Harrison (169) has found complete return to normal levels of amino acid excretion after treatment of rickets. Aminoaciduria has also been reported in vitamin-E-deficient animals (170).

Storage diseases and errors of metabolism—Most of the papers which have appeared on hemochromatosis during the past year reaffirm the usual histologic findings and the occurrence of large amounts of iron in various organs (171, 172). Wishinsky and associates (173) have reported that the administration of the chelating agent, EDTA, to patients with hemochromatosis results in a greatly increased urinary excretion of iron. Although it was originally believed that the abnormal lipide storage in Gaucher's disease consisted of a cerebroside which contained galactose as its constituent sugar, several reports about 15 years ago indicated the presence of glucose in some instances. Recent chromatographic studies of the lipides of spleens from patients with this disease show that lactose, surcose, or fructose may also be found in these lipides (174, 175).

The group of "inborn errors of metabolism" includes a number of rare diseases which have a familial tendency and are characterized by a biochemical abnormality, attributable to the absence of one or more enzymes from the usual metabolic sequence or by a reabsorptive defect in the renal tubules. Black *et al.* (176) have recently reported five cases of alcaptonuria and ochronosis occurring in one family, have traced the inheritance of the abnormality in this family, and have summarized the biochemical determinations of use in such studies. Dent *et al.* (177, 178) have continued their studies on cystinuria; determination of the plasma pattern of blood amino acid levels and of the urinary amino acid excretion after administration of L-cystine, DL-methionine, and L-cysteine have led them to conclude that the fault lies in renal tubular reabsorption of cystine rather than in a disorder of the intermediary metabolism of sulphur-containing amino acids. Cystinosis is a rare disease characterized by deposition of cystine in many different tissues and by death in early life. The first cases to be found in the United States were reported in 1953 by Jackson & Clarke (179), and the various changes in blood biochemical parameters were described. Dent & Philpot (180) have recently added another case of urinary xanthine stone to the 23 cases previously reported. The urine contained large amounts of xanthine but negligible amounts of uric acid; the plasma, on chromatographic analysis, did

not show either xanthine or uric acid. This patient either lacked liver xanthine oxidase or else had a renal impairment whereby xanthine was excreted so rapidly that it could not be metabolized to uric acid. Nitowsky *et al.* (181) have shown that the blocks in the oxidation of phenylalanine and tyrosine, with the resultant phenyluria, that are present in premature infants are corrected by various hemopoietic and other factors. Such correction may indicate the existence of an alternate path for the degradation of the deaminated residues of tyrosine and phenylalanine.

Gout.—In a metabolic study of two patients with gout, Levin and associates (182) did not observe any regular association of sodium and chloride diuresis with gouty arthritis and thus failed to confirm the concept of a "gouty cycle." Studies on the utilization of glycine-N^{15} in uric acid synthesis by gouty patients continue to accumulate (183, 184). It would appear that these patients fall into two types: those who synthesize uric acid very rapidly and, provided that the renal function is normal, excrete large amounts in the urine; and second, those who are within normal limits in these respects.

Diseases of calcium and phosphorus metabolism.—Several procedures have been proposed to evaluate the calcium and phosphorus metabolism at various stages of diseases affecting the skeleton. Howard and associates (185) have advocated an intravenous calcium injection to aid in diagnosis of parathyroid states. Laszlo *et al.* (186) and Pearson and associates (6) have suggested the measurement of urinary calcium excretion as an index of the growth of breast cancer metastatic to the skeleton. Griboff and co-workers (187) showed that an inverse relationship between changes in serum calcium concentration and serum alkaline phosphatase activity exists in patients with hypercalcemia attributable to bone metastases from the breast.

The various biochemical features of the rare Fanconi syndrome, namely, decreased calcification, hypophosphatemia, hyperphosphatasemia, renal glycosuria, and aminoaciduria, have received further study (188, 189). In the case reported by Kyle *et al.* (188), the underlying mechanism appeared to be a tubular reabsorption defect for phosphate. Sirota & Hamerman (189) found that the depressions in renal tubular functions were significantly greater than the decreases in glomerular filtration.

Interest has been revived in the mineral components of bone, other than calcium and phosphorus, and the extent to which they may be metabolically involved in human disease. Bergstrom (190) showed that the sodium and potassium content of rat bone could be readily depleted by intraperitoneal dialysis. Edelman and associates (191) have obtained mean values of 12,490 m. eq. of calcium, 234 m. eq. of sodium, 198 m. eq. of excess sodium, and 29 m. eq. of chloride per kg. of fat-free bone solids in man. Experiments with Na^{22} and Na^{24} showed that about 45 per cent of bone sodium was exchangeable in about 4 hr.

Other Diseases

Diseases of muscle.—Caldwell & Prankerd (192) have added to the few

available values on the concentration of organic phosphate esters in normal and diseased human muscle. In myotonia congenita the creatine phosphate was found to be low and the orthophosphate and monophosphate high, at rest and during myotonia, but tended to return to the levels of normal resting muscle when the myotonia was worked off. The method of expressing muscle constituents in terms of noncollagenous protein nitrogen (NCN), which was developed by Baldwin *et al.* (193), has been applied by Dreyfus and associates (194), who found greatly increased values for NCN in muscular dystrophy and very low values for phosphorylase "A," phosphorylase "B," and aldolase per gram NCN.

Studies of the urinary excretion of ribose in various myopathies continue to attract attention, but the reported results are not consistent, possibly because of the use of different biochemical tests. Orr & Minot (195) obtained uniformly positive results in progressive muscular dystrophy, dystrophia myotonica, myotonia congenita, and amyotonia congenita, but not in myasthenia gravis, secondary muscular atrophies, or in other neuromuscular diseases. Drew & Selving (196) also reported similar results.

Using a paper chromatographic method sensitive to 5 mg. ribose per 100 ml. of urine, Matthews & Smith (197) were not able to obtain any positive tests for ribose in six cases of progressive muscular dystrophy, two cases of dystrophia myotonica, and in three other myopathies. Walton & Latner (198) obtained 12 positive tests for ribosuria in 89 cases of myopathy and two positive tests in five cases of motor neurone disease.

Psychiatry.—Within the past few years several chemical compounds, such as mescaline and D-lysergic acid diethylamide tartrate (LSD), have been found to produce reversible schizophrenia-like symptoms in man, the latter in very small doses of about 1 μg. per kg. [Rinkel *et al.* (199); Hoch and associates (200); Savage (201)]. In addition to this property they are of interest because they may be related to other metabolically important compounds.

Gaddum and co-workers (202) observed that the vasoconstrictive and bronchoconstrictive actions of serotonin [3-(β-amino-ethyl)-5-hydroxyindole] on cats' lungs were antagonized by the structurally related LSD[2]. Wooley & Shaw (203) demonstrated that a number of synthetic compounds, structurally related to serotonin, antagonized in a competitive manner the arterial wall contractions induced by serotonin and observed that 2-methyl-3-ethyl-5-dimethyl-aminoindole, a highly active synthetic antimetabolite of serotonin, caused epileptiform convulsions in mice (204). They have suggested that serotonin possibly plays a role in maintaining normal mental processes, that some mental disorders may be a reflection of metabolically induced deficiency of serotonin, and that serotonin or a long-acting derivative of it may prove of value in alleviating disorders similar to schizophrenia. Meyers (205) has pointed out that the pharmacological properties of serotonin, 2-methyl-3-ethyl-5-dimethyl-aminoindole, and other antagonists are not sufficiently specific to permit inferences about the genesis of disease entities.

Recently Fischer (206) has summarized his work on the biochemical factors involved in reversible drug-produced model psychoses. Considering wool protein as a model for the protein component of the target organ, he has shown that the absorption of such drugs by protein is inversely proportional to the doses in which LSD, lysergic acid monoethylamide, mescaline, and other drugs produce the experimental psychoses in man. Fischer's formulations involve a number of assumptions and speculative features, but their general trend is in line with current pharmacological investigations on the biochemical bases for drug action.

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LIPIDE METABOLISM[1,2]

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A review of lipide metabolism by Artom (1) was published in 1953. Since then the details of the process of β-oxidation, whereby the stepwise conversion of fatty acid molecules into C_2 units is effected, have been explained. Fifty years ago Knoop demonstrated its importance by feeding experiments. The two discoveries leading to this ultimate explanation were the discovery of coenzyme A and, in connection with it, the discovery of energy-rich acyl-mercaptan- or thioester compounds. The intermediate products in the biological synthesis and degradation of the fatty acid chain which had been sought for a long time prove to be thioesters of CoA.

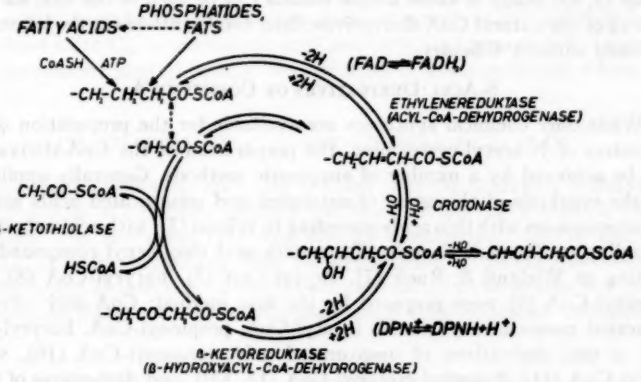


FIG. 1. Role of CoA in the metabolism of fat. (The fatty acid cycle.)

Our present picture of the role of CoA in the metabolism of fat is to be seen in Figure 1. It is a cyclic process in which all steps of the reaction are reversible such that synthesis and degradation of fatty acid chains may

[1] The survey of the literature pertaining to this review covers the period December, 1952 to December, 1954.

[2] The following abbreviations are used: Coenzyme A, CoA or CoASH; S-acyl coenzyme A derivatives, acyl CoA, S-Acyl CoA, or acyl-SCoA; adenosine mono-, di-, and triphosphate, AMP, ADP, and ATP; guanosine di-, and triphosphate, GDP, GTP; inosine di- and triphosphate, IDP, ITP; pyrophosphate, PP; orthophosphate, Pi; oxidized and reduced diphosphopyridine nucleotide, DPN^+ and DPNH; oxidized and reduced triphosphopyridine nucleotide, TPN^+ and TPNH; oxidized and reduced flavin adenine dinucleotide, FAD and $FADH_2$; flavin mononucleotide, FMN.

equally be achieved. For this process the name fatty acid cycle or fatty acid spiral has been proposed (2).

By conversion into the CoA derivative the fatty acid is "activated" and thereupon yields acetyl-CoA and an acyl-CoA with two carbon atoms less than the starting substance. This is subjected immediately to the same degradation sequence in a second cycle, again liberating acetyl-CoA, and so on. It is essentially the sequence of reactions already formulated by Dakin (3), with CoA derivatives involved instead of free acids. It was substantially due to the preparation of the specific substrates for these enzymes that the individual enzymes of this cyclic process could be identified. The preparation of the specific substrates made possible the various enzyme tests which allowed the study of the enzymes.

This goal was achieved in two ways: one by the development of preparative methods pertaining to the production of the various thioesters of CoA; the other by making use of simple models wherein the acids are linked only with cysteamine, the fragment of CoA carrying the functional SH group (2, 4). Many of these simple models can be used in the enzyme tests instead of the natural CoA derivatives; their total synthesis in the laboratory proceeds without difficulty.

S-Acyl Derivatives of Coenzyme A

While only chemical syntheses are available for the preparation of the thioesters of N-acetyl-cysteamine, the preparation of the CoA derivatives can be achieved by a number of enzymatic methods. Generally applicable for the synthesis of thioesters of saturated and unsaturated acids are the transformations with thio acids according to Wilson (5), with acid anhydrides according to Simon & Shemin (6), or with acyl thiophenyl compounds according to Wieland & Rueff (7). Acetyl-CoA (5), butyryl-CoA (8), and palmityl-CoA (9) were prepared by the first method; CoA derivatives of saturated monocarboxylic acids (acetyl-CoA, propionyl-CoA, butyryl-CoA and so on), derivatives of unsaturated acids [crotonyl-CoA (10), vinylacetyl-CoA (11), β-methyl-crotonyl-CoA (12, 13)], and derivatives of polybasic acids [succinyl-CoA (6) or β-hydroxy-β-methyl-glutaryl-CoA (14)] were obtained with the second technique. Hydroxy-acyl and β-ketoacyl-CoA derivatives are accessible by the procedure of Wieland & Rueff (7, 13, 203). But for the simplest members of this series, β-hydroxybutyryl-CoA or acetoacetyl-CoA, the best method of preparation is the reaction of CoA with β-butyrolactone (15) or with diketene (15, 16), respectively.

Both CoA derivatives may also be obtained enzymatically: acetoacetyl-CoA by enzymatic CoA-transfer from synthetic succinyl-CoA to acetoacetate (17) (reaction 22), or by enzymatic dehydrogenation of β-hydroxybutyryl-CoA (18, 19) (reaction 10). The latter method can equally be applied for the synthesis of homologous β-ketoacyl-CoA compounds (19). The necessary β-hydroxyacyl-CoA derivatives may be obtained from the free acids, CoASH, and ATP in the presence of the special "activating enzyme." This reaction

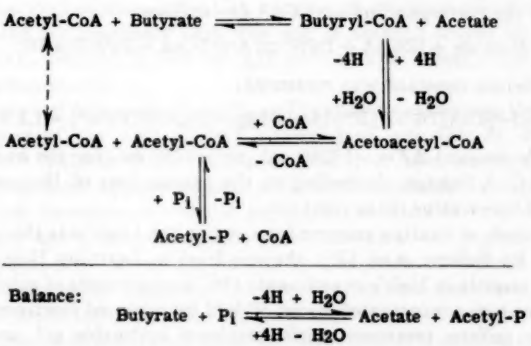
was also adopted for the synthesis of CoA derivatives of saturated and unsaturated carboxylic acids between C_2 and C_{12} (19, 20, 21) and of palmityl-CoA (22). Another method for the preparation of acetyl-CoA and succinyl-CoA is the enzymatic dehydrogenation of pyruvate (23) and α-ketoglutarate, respectively (24, 25). Succinyl-CoA may also be obtained by the enzymatic reaction between succinate, CoA, and ATP (25) (reaction 24). Bacterial enzymes have also been employed successfully for preparative purposes. Stadtman described the enzymatic synthesis of acetyl-CoA from acetyl-phosphate and CoA with phosphotransacetylase (26, 27) and of butyryl-CoA from acetyl-CoA and butyrate with the cooperation of CoA-transphorase (8) (reaction 1).

Fatty Acid Activation

CoA-transphorase.—The function of CoA-transphorase from *Cl. kluyveri* is, in part at least, the activation of higher fatty acids. Stadtman (8) has thoroughly studied the enzyme in cell-free extracts. It survived heating at 60° for half an hour and could thus be freed of phosphotransacetylase. The enzyme catalyses the reversible CoA transfer from acetyl-CoA to normal fatty acids from C_1 to C_5, to vinylacetic acid and lactic acid (reaction 1). Caprylic acid, acetoacetic acid, β-hydroxybutyric acid, crotonic acid and others are inactive.

$$CH_3 \cdot CO{-}SCoA + R \cdot COO^- \rightleftharpoons R \cdot CO{-}SCoA + CH_3 \cdot COO^- \qquad 1.$$

CoA-transphorase is responsible for the fact that the oxidation of butyrate in extracts of *Cl. kluyveri* proceeds without further help after a single "ignition." As Stadtman (28) was able to demonstrate, one of the two acetyl-CoA units originating from the degradation of butyrate is used for the activation of butyrate, while the second reacts in the presence of phosphotransacetylase with orthophosphate and thus produces acetylphosphate.



Acetokinase.—Microorganisms containing CoA-transphorase and phosphotransacetylase in general possess also an enzymatic catalyst for the reaction:

$$\text{Acetate} + \text{ATP} \rightleftarrows \text{Acetyl P} + \text{ADP} \qquad 2.$$

According to Rose, Grunberg-Manago, Korey & Ochoa (29), who studied the purified enzyme of *S. haemolyticus* and *E. coli*, propionate is converted as well as acetate; Mg^{++} or Mn^{++} is indispensable for the catalysis. From the equilibrium constant, $K = [\text{ADP}][\text{Acetyl-P}]/[\text{ATP}][\text{Acetate}]$, which equals 0.01 (pH 7, 29°), ΔF is calculated to be -3000 cal. Experiments with labeled acetate and labeled ADP show that phosphate is transferred directly from ATP to acetate and that there is no intermediate enzyme-phosphate binding. This enzyme, called acetokinase by Rose *et al.* (29), provides in bacteria containing phosphotransacetylase the linkage between acetyl-CoA and ATP.

Acetate-activating enzyme.—In animal tissues, yeast, plants, and in *Rhodospirillum rubrum* formation of acetyl-CoA occurs through pyrophosphate cleavage from ATP. The detailed description of the experiments in which Jones *et al.* (30) discovered this is now available. In the presence of fluoride, which inhibits the pyrophosphatase still adhering to the enzyme preparation, the balance was according to the equation:

$$\text{ATP} + \text{Acetate} + \text{HSCoA} \rightleftarrows \text{Acetyl—SCoA} + \text{AMP} + \text{PP} \qquad 3.$$

The purified enzyme required Mg^{++}. As equilibrium constant, Jones (31) found $K = [\text{Acetyl-CoA}][\text{AMP}][\text{Pyrophosphate}]/[\text{Acetate}][\text{ATP}][\text{CoA}] = 2.7$. This confirms the approximate energy equivalence of the acylmercaptan bond in acetyl-CoA and the pyrophosphate bond of ATP, as already established previously by measurements of Stadtman (26) as well as Stern, Ochoa & Lynen (32).

Investigations by Burton & Stadtman (33) on purified aldehyde dehydrogenase of *Cl. kluyveri* are of importance in this connection. This enzyme catalyzes the reversible oxidation, linked to DPN, of acetaldehyde, propionic aldehyde, butyric aldehyde or glycolic aldehyde, in the presence of CoA, to the corresponding acyl-CoA derivatives.

$$\text{Aldehyde} + \text{HSCoA} + \text{DPN}^+ \rightleftarrows \text{Acyl-SCoA} + \text{DPNH} + \text{H}^+ \qquad 4.$$

The equilibrium constant was measured:

$$K = [\text{Acetyl—SCoA}][\text{DPNH}][\text{H}^+]/[\text{Acetaldehyde}][\text{CoASH}][\text{DPN}^+] = 1{,}2 \times 10^{-4},$$

from which resulted $\Delta F = -13040$ cal. or -8250 cal. for the hydrolysis of the acetyl-CoA linkage. According to the calculations of Burton (34) the second and lower value is the right one.

The acetate-activating enzyme from rat or hog heart was thoroughly investigated by Beinert *et al.* (35), the one from ox heart by Hele (36). The starting materials in Hele's experiments (36) were extracts of mitochondria. The enzyme was concentrated about 30-fold by repeated fractionation with ammonium sulfate, treatment with aluminum hydroxide gel, and electrophoresis. With the pure, electrophoretically homogeneous enzyme, only

propionate and acrylate, besides acetate, were converted into the corresponding acyl-CoA derivative (reaction 3, equilibrium constant in the proximity of 1) (36). The investigation of this enzyme was made very difficult by its increasing instability with progressive purification.

Hilz (37) had to contend with the same difficulty while purifying the acetate-activating enzyme from yeast maceration juice. Approximately 60-fold concentration was achieved by a procedure which included fractionation with ammonium sulfate at alkaline pH and ethanol fractionation at 0° in the presence of Zn^{++} and cysteine. Hilz (37) came to the same result as the investigators in Madison (35, 36) and in Boston (30), according to which only one single enzyme and Mg^{++} participate in this reaction.

The mechanism could be explained by experiments with radioactively labelled compounds (39). The enzyme reacts therein successively with the various substrates. The 3 steps are (E = enzyme):

$$E + ATP \rightleftharpoons E\text{—}AMP + PP \qquad 5.$$

$$E\text{—}AMP + HSCoA \rightleftharpoons E\text{—}SCoA + AMP \qquad 6.$$

$$E\text{—}SCoA + Acetate \rightleftharpoons E + Acetyl\text{—}SCoA \qquad 7.$$

The experimental findings leading to this formulation are: (*a*) ATP rapidly reaches an equilibrium with P^{32}-labelled pyrophosphate in the absence of CoA (reaction 5); (*b*) this exchange reaction is inhibited by CoA, as a consequence of the formation of E-SCoA (reaction 6) with a simultaneous lowering of the concentration of E-AMP decisive for the exchange; and (*c*) incorporation of C^{14}-labelled acetate into acetyl-CoA, also in the absence of AMP (reaction 7). It resulted furthermore that Mg^{++} is only necessary for reaction 5.

According to Seaman (40) lipoic acid should participate in the acetate activation, which can be inhibited by As_2O_3. Extracts of pigeon liver (acetone powder) free of CoA, having lost a large amount of their activity through treatment with alumina, could be reactivated by lipoic acid.

The purified acetate-activating enzyme of *Rhodospirillum rubrum* was investigated by Eisenberg (41), the one of spinach leaves by Millerd & Bonner (42). Millerd & Bonner (42) were able to identify the enzyme in various green plants.

Activation of fatty acids, C_4 to C_{11}.—The enzyme catalyzing the formation of thioesters of the acids C_4 to C_{11} (analogous equation 3) was isolated by Mahler, Wakil & Bock (21) from beef liver mitochondria. The commonly used interception method with hydroxylamine for the detection of acyl-CoA formation could not be employed since the purified enzyme is damaged by hydroxylamine in high concentration. Mahler *et al.* used as the method of determination the consumption of CoASH by means of the nitroprusside reaction. The purified enzyme activates the normal saturated acids (optimum at C_7), acids with double bonds in the α:β- or β:γ-position, α- or β-hydroxy acids and acids with a branching carbon chain such as β- or γ-methyl valeric acid. β-Keto or α-amino acids either do not,

or only slowly, react with the enzyme, though aromatic compounds like benzoic acid, phenylacetic acid, and 2,4-dichlorophenoxy acetic acid do. For the equilibrium constant of the enzymatic reaction (analogous equation 3) a value in the proximity of 1 was found which makes it probable that the diverse acyl-CoA derivatives are energetically equivalent. For these measurements Mahler *et al.* (21) made use of the elegant method of Stadtman (27) where formation and cleavage of the thioester is observed optically at 232 to 240 mμ.

The formation of CoA esters of saturated fatty acids (43), crotonic acid, vinylacetic acid, and D- as well as L-hydroxybutyric acid (44) was also established in extracts of rat liver mitochondria. An enzyme of similar specificity was found by Millerd & Bonner (42) in spinach leaves.

Activation of higher fatty acids.—Kornberg & Pricer (22) discovered an enzyme in extracts of guinea pig liver which converts higher fatty acids of C_6 to C_{22}, including unsaturated acids (linolic, linolenic, oleic, and chaulmoogric acid), according to equation 3, into the corresponding acyl-CoA derivatives. The optimum activity of the crude enzyme preparation was with lauric acid (C_{12}). On homogenization of the liver the enzyme went partly into solution; the remainder was combined with particles sedimenting at 110,000×g. Preparations from rat-, mice-, pigeon-, and chicken livers were substantially less active. Other organs, as well as extracts from yeast, *E. coli*, or *Cl. kluyveri* contained practically no activity.

Enzymes of the Fatty Acid Cycle

Ethylene reductase, butyryl coenzyme A dehydrogenase, fatty acyl CoA dehydrogenase.—The reversible dehydrogenation of fatty acid CoA compounds takes place according to the equation:

$$R \cdot CH_2 \cdot CH_2 \cdot CO{-}SCoA + FAD \rightleftharpoons R \cdot CH{:}CH \cdot CO{-}SCoA + FADH_2 \qquad 8.$$

Three different enzymes of this type have been discovered in animal liver. Seubert & Lynen (45) isolated an enzyme, ethylene reductase, from sheep liver by making use of the fact that this enzyme also converts derivatives of N-acetyl cysteamine instead of the natural CoA derivatives. As an enzyme test they employed the enzymatic oxidation of leucosafranin by S-crotonyl-N-acetyl cysteamine. The solution of the FAD protein which was concentrated 400-fold, as compared to the crude extract, was of a clear yellow color with absorption maxima at 275, 362, and 445 mμ (ratio of the extinctions 9.4:0.9:1.0) (46, 53). The colorless apoenzyme obtained by precipitation with ammonium sulfate at pH 3.7 to 3.9 could be reactivated with FAD or larger amounts of FMN (53). The substrate specificity of the purified enzyme proved to be rather broad: besides S-crotonyl-CoA, S-octenoyl-CoA and S-β-methylcrotonyl-CoA were reduced in the test system, although at a slower rate than the reference substance. It was possible to study the activity of the enzyme in the reverse direction with 2,6-dichlorophenolindophenol as hydrogen acceptor, whereby thioesters of N-acetyl cysteamine could equally

well be used as substrate. The findings showed a rise in the rate of reaction in the succession of derivatives of normal fatty acids from C_4 to C_8 (46, 53).

Isolation of a similar enzyme from beef liver mitochondria which also contained FAD was reported by Beinert & Crane (47). The 60-fold enriched enzyme, called "Fatty acyl CoA dehydrogenase" by the authors, showed absorption maxima at 275, 375, and 445 mμ (ratio of the extinctions: 9.4:0.8:1.0). Upon addition of CoA derivatives of fatty acids C_4 to C_{16}, the canary yellow color of the solution of the purified enzyme was bleached on account of immediate reduction. The iron in the purified enzyme is regarded by Beinert & Crane (47) as an essential constituent. They support this with the facts that: (*a*) in preparations with differing degree of purity the proportion iron:flavine constantly was found as 1:3; and (*b*) that in reduction of the enzyme by CoA-thioesters an absorption band appeared between 520 and 600 mμ. DPNH-cytochrome-*c*-reductase, another iron-flavoprotein, showed in reduced state an absorption band in the same spectral region, according to findings in Green's laboratory (48).

Besides this fatty acyl-CoA dehydrogenase a butyryl coenzyme A dehydrogenase of green colour could be isolated from beef liver mitochondria in Madison (49). It reacts only with CoA derivatives of the fatty acids C_3 to C_8 (optimum C_4). The relatively high electrophoretic mobility of the enzyme made it possible to introduce electrophoresis as an efficient purifying step and thus to obtain a total 250-fold concentration of the enzyme. The purified enzyme proved homogeneous in the ultracentrifuge; the molecular weight was 120,000 to 220,000. Optical enzyme tests in the presence of butyryl-CoA were (*a*) decoloration of 2,6-dichlorophenolindophenol; (*b*) formation of formazan from triphenyltetrazolium chloride in the presence of pyocyanine; and (*c*) reduction of cytochrome-*c*. The Michaelis constant for butyryl-CoA in the indophenol test was found as $K_m' = 1.4 \times 10^{-5}$ (moles /liter); the equilibrium constant $K = [\text{Butenoyl-CoA}][\text{Leucoindophenol}] / [\text{Butyryl-CoA}][\text{Indophenol}] = 3$ to 22. E_0' was derived for butyryl-CoA/butenoyl-CoA and was equal to 0.187 volt (pH 7.0, 25°), a value considerably above the potential of butyrate/crotonate with $E_0' = -0.025$ volt. The inhibition of the enzyme by *p*-chloromercuribenzoate is noteworthy and indicates a participation of SH-groups in the enzymatic reaction. Mahler (50) found the four band absorption spectra of the enzyme with maxima at 265, 355, 432.5, and 685 mμ to be dependent upon the content of FAD and Cu^{++}, which are bound in the ratio of 1:2. The apoenzyme was obtained by cleavage with ammonium sulfate at pH 3.6 to 4.1. It could be reactivated by FAD but not by FMN. Dialysis of the enzyme against a solution of KCN led to the splitting off of copper and simultaneous disappearance of the unusual flavoprotein absorption maximum at 685 mμ. An investigation of the enzymatic behaviour of preparations containing copper and without copper showed the metal to be participating neither in the reduction of the flavine by the substrate nor in the reaction between leucoflavine and 2,6-dichlorophenolindophenol (2 electron acceptor!). The presence of copper was

of no importance for the kinetics of either process, but the capacity to reduce cytochrome-*c* or $K_3Fe(CN)_6$ (1 electron acceptor!) was substantially slowed down with the metal-free enzyme; from this Mahler concluded that copper facilitates the transport of electrons from the reduced flavine to 1-electron acceptors (50, 51).

According to a preliminary communication by Crane & Beinert (52) the conditions seem to be still more involved. These authors report on preparations of their fatty acyl-CoA dehydrogenases from hog liver, purified by electrophoresis, which after reduction by the specific substrates were not reoxidizable by 2,6-dichlorophenolindophenol or by cytochrome-*c*. This oxidation necessitated a new FAD flavoprotein (absorption maxima: 270, 375, and 440 mμ; ratio of extinctions: 6.5:0.9:1.0) for which the name "cytochrome-*c* reducing factor" was proposed. The fact that this enzyme, in which so far no metal could be detected, loses its activity with cytochrome-*c* after some time but not with dichlorophenolindophenol, indicates the reduction of cytochrome-*c* to be a complex process.

Our knowledge about the supply of the hydrogen necessary for the hydrogenation of α:β-unsaturated acyl-CoA derivatives *in vivo* is likewise insufficient. Its origin in reduced pyridine nucleotides is probable, but enzymes that facilitate hydrogen transport from DPNH or TPNH to ethylene reductase are unknown at this time.

Crotonase, unsaturated fatty acyl coenzyme A hydrase.—Stern & Del Campillo (10) found crude enzyme preparations from heart or liver which also contain the enzyme for the oxidation of β-hydroxybutyryl-CoA, able to convert synthetic S-crotonyl-CoA into S-acetoacetyl-CoA in the presence of DPN. On the other hand, Seubert & Lynen (45) noticed that crude preparations of ethylene reductase, instead of crotonyl-CoA, also reduced β-hydroxybutyryl-CoA with leucosafranine. Both observations favour the existence of an enzyme catalyzing reaction 9, for which the name crotonase (10) was proposed.

$$R \cdot CH{:}CH \cdot CO{-}SCoA + H_2O \rightleftarrows R \cdot CH(OH) \cdot CH_2 \cdot CO{-}SCoA \qquad 9.$$

Besides these indirect methods of detection of the crotonase, which allowed the spectrophotometric determination of the enzymes when combined with highly purified ethylene reductase or β-ketoreductase preparations (53, 54), a direct enzyme test has been developed in Ochoa's laboratory (16). In this test use has been made of the absorption band of crotonic acid thioester at 263 mμ (45).

Wakil & Mahler (54) concentrated the enzyme from extracts of beef liver mitochondria about 17-fold and called it "unsaturated fatty acyl coenzyme A hydrase." The purified enzyme showed a wide specificity of activity. Crotonyl-CoA (but not isocrotonyl-CoA), trans hexenoyl-CoA, as well as the CoA thioesters of normal C_4, C_6, C_8, and C_{12} β-hydroxy acids were converted. The observation that also vinylacetyl-CoA is hydrogenated by the enzyme to β-hydroxybutyryl-CoA is of special importance. Thus, it is possible

that through the β-hydroxy compound an isomerization of the double bond takes place from α:β to β:γ and vice versa, a process which could be of importance to the degradation or the synthesis of naturally-occurring unsaturated fatty acids, as, for example, oleic acid and others. It would then be expected, of course, that in the series of β:γ-unsaturated acid derivatives the *cis* isomers would also be converted by crotonase.

The equilibrium between crotonyl, vinylacetyl and β-hydroxybutyryl-CoA is in favour of the formation of hydroxy compounds: Wakil & Mahler indicate the ratio of β-hydroxy- to dehydroacyl-CoA as being 1.40 to 1.58. From the fact that crotonyl-CoA, in the presence of DPN, crotonase, and β-ketoreductase is converted quantitatively into acetoacetyl-CoA it follows that by the stereospecific enzymatic process of hydrogenation D-β-hydroxybutyryl-CoA is formed (54).

The strict proof of the fact, that all these activities are attributable to one single enzyme was provided by Stern, Raw & Del Campillo (11) with the crystallization of crotonase from beef liver. The crystallized enzyme (molecular weight 120,000) shows with crotonyl-CoA the considerable "turnover number" of 420,000; it also hydrogenates β-methyl crotonyl-CoA and sorbyl-CoA, but is inactive with free crotonic acid and S-crotonyl glutathione, as Stern *et al.* observed. In the Munich laboratory (55) it was found, making use of a purified enzyme fraction from sheep liver, that crotonase was the only enzyme of the fatty acid cycle which showed no effect with thioesters of N-acetyl cysteamine, pantetheine, or pantetheine-4′-phosphate.

β-Ketoreductase, β-hydroxyacyl coenzyme A dehydrogenase.—Lynen *et al.* (18) reported the presence of an enzyme which catalyzes a reversible reaction between acetoacetyl-CoA and DPNH (equation 10) in sheep liver. They proposed for it the name β-ketoreductase.

$$R \cdot CHOH \cdot CH_2 \cdot CO\text{—}SCoA + DPN^+ \rightleftharpoons R \cdot CO \cdot CH_2 \cdot CO\text{—}SCoA + DPNH + H^+ \quad 10.$$

Stern, Coon & Del Campillo (17) discovered a similar enzyme in extracts of heart muscle. Experiments by Lehninger & Greville (44) produced evidence that this enzyme is specifically oriented towards D-β-hydroxyacyl-CoA esters. They observed two enzyme systems in extracts of rat liver mitochondria for the oxidation of β-hydroxybutyrate by DPN. One of them was identical with the known L-β-hydroxybutyric dehydrogenase while the other needed the presence of ATP as well as CoA and was oriented specifically to D-β-hydroxybutyrate. The authors were able to prove that, in this case (analogous to reaction 3) and with the participation of ATP, D-β-hydroxybutyryl-CoA was formed first, which then was subjected to oxidation by the specific dehydrogenase. L-β-Hydroxybutyrate also was converted to the CoA derivative but this could not be oxidized.

The purification from liver of β-ketoreductase, an enzyme for which the name β-hydroxyacyl coenzyme A dehydrogenase is also in use, was carried out by researchers in Munich (18, 57), and in Madison (56). Lynen *et al.* (18) took advantage of the fact that β-ketoreductase also reacts with deriva-

tives of N-acetylcysteamine in their enzyme test and observed the consumption of DPNH by S-acetoacetyl N-acetylcysteamine.

Lynen & Wieland (57) studied the kinetics of reduction of various S-acetoacetyl-cysteamine derivatives making use of a highly purified enzyme preparation. In high concentrations of the substrate the maximal rate for the various derivatives was almost equal. Half maximal rates were established with 0.11 μMol/ml. for S-acetoacetyl-CoA, 0.22 μMol/ml. for S-acetoacetyl-4'-pantetheine phosphate, 2.2 μMol/ml. for S-acetoacetyl-pantetheine and 11 μMol/ml. for S-acetoacetyl-N-acetylcysteamine (pH 7.6, 25°). Further extension of these experiments may offer a means of understanding the biological significance of the specific structure of coenzyme A.

In respect to the acid residue linked to the SH groups of coenzyme A or N-acetylcysteamine the enzyme shows a wide specificity. All tested derivatives of the β-hydroxy acids from C_4 to C_{12} were converted (56, 57). The equilibrium constant for reaction 10 was found to be consistent with CoA- and N-acetylcysteamine derivatives (58, 59):

$$K = [\text{D-}\beta\text{-hydroxybutyryl-CoA}][\text{DPN}]/[\text{Acetoacetyl-CoA}][\text{DPNH}][\text{H}^+] = 5.25 \times 10^8 (25°).$$

This value is applicable between pH 5.8 and about 8.5. Above 8.5 an additional effect is observed with salt formation by the acetoacetyl thioesters displacing the equilibrium practically completely to the side of the formation of keto acid. This fact has been utilized for the preparation of acetoacetyl-CoA and its homologues (18, 19).

Distribution of β-ketoreductase in the animal and human organism was investigated in Munich (57, 60). It is noteworthy that heart muscle has a high enzyme activity and brain a slight one. The enzyme could also be detected in various butyric acid bacteria (28, 61, 62).

β-Ketothiolase, acetoacetate condensing enzyme, β-ketoacyl coenzyme A cleavage enzyme.—The enzymes of this class which catalyze the general re- tion 11 bring about the key reactions of degradation and synthesis of the fatty acid chains.

$$RCH_2 \cdot CO \cdot CH_2 \cdot CO\text{—SCoA} + HSCoA \rightleftharpoons RCH_2 \cdot CO\text{—SCoA} + CH_3 \cdot CO\text{—SCoA} \quad 11.$$

The biological significance of the participation of sulphur consists in the fact that the thiolytic cleavage in contrast to the hydrolytic cleavage is reversible.

The observation that the acetoacetate synthesis from acetate, catalyzed by liver enzymes, requires two activated C_2 units (63), the findings of Barker *et al.* (64) on the cleavage of β-keto acids by soluble enzymes from *Cl. kluyveri*, and also, the necessity of an "activation" of the acetoacetate by ATP in the formation of citrate (65) make reaction 11 and the existence of a corresponding enzymatic catalyst very probable (64, 66).

The first direct discovery of this enzyme, for which the names β-ketothiolase (18), acetoacetate condensing enzyme (17), and β-ketoacyl coenzyme A cleavage enzyme (67) have been proposed, was successful in combined enzyme tests, where CoA transferase (reaction 12) with citrate

condensing enzyme (67a, 68) (reaction 14) or β-ketoreductase (18) were active as auxiliary enzymes. Stern *et al.* (67a), as well as Green *et al.* (68), found that in extracts of hog's heart, citrate is formed from acetoacetate and oxaloacetate when succinyl-CoA is present. This process may be explained by the equations:

$$\text{Succinyl-CoA} + \text{Acetoacetate} \rightleftharpoons \text{Acetoacetyl-CoA} + \text{Succinate} \qquad 12.$$

$$\text{Acetoacetyl-CoA} + \text{CoA} \rightleftharpoons 2\ \text{Acetyl-CoA} \qquad 13.$$

$$\text{Acetyl-CoA} + \text{Oxaloacetate} \rightleftharpoons \text{Citrate} + \text{CoA} \qquad 14.$$

Lynen *et al.* (18) demonstrated the formation of acetoacetyl-CoA from acetyl-CoA, in the presence of an enzyme fraction from sheep liver, by enzymatic reduction to β-hydroxybutyryl-CoA.

Investigation of the thiolase reaction (2, 16, 17, 67), and also of the CoA transferase reaction (16, 17), was considerably facilitated by the observation that thioesters of β-keto acids show a strong absorption band at 303 mμ (18) in alkaline solution, which can be intensified by addition of Mg^{++} (17). Mg^{++} forms a complex (1 Mg^{++}:2 thioester) which in case of the S-acetoacetyl-N-acetylcysteamine could be obtained in crystalline form and was studied thoroughly by Lynen & Decker (70).

As a routine test in the purification of β-ketothiolase from hog heart muscle, Ochoa's laboratory (16) has used the optical determination at 303 mμ of the thiolytic cleavage of acetoacetyl-CoA by CoA. The purified enzyme fraction, concentrated 300-fold as compared with the crude extract, proved homogeneous in the ultracentrifuge and catalyzed the cleavage of 7000 to 8000 moles of acetoacetyl CoA/min./100,000 gm. protein (25°) (69). The purified enzyme reacted only with acetoacetyl-CoA and, essentially more slowly, with β-ketovaleryl-CoA, but not with β-ketocaproyl- or β-ketoisocaproyl-CoA. The fact that the higher ketoacyl derivatives were split by crude heart muscle extracts (67a, 68), as well as by a concentrated enzyme fraction from beef liver mitochondria (67), permits one to conclude that various thiolases occur in the animal organism (16) and cooperate in the degradation and synthesis of higher fatty acids. Goldman (67) used a combined test (with crotonase, β-ketoreductase, malicodehydrogenase, and citrate-condensing enzyme as auxiliary enzymes) for the purification of β-ketothiolase, based upon the over-all reaction:

$$\text{Crotonyl-CoA} + 2\text{L-malate} + 3\ \text{DPN}^+ + 3\text{H}_2\text{O} \rightarrow 2\ \text{Citrate} + 3\ \text{DPNH} + 3\text{H}^+ + \text{CoA} \qquad 15.$$

Lynen, Decker & Reinwein (4, 73) studied the effective range of the enzyme with regard to the mercaptan component in a β-ketothiolase preparation from sheep liver. Besides S-acetoacetyl-CoA (Michaelis constant: 2.2×10^{-5} mole/liter, 25°), S-acetoacetyl-pantetheine (3.0×10^{-5}), S-acetoacetyl-pantetheine-4′-phosphate (10×10^{-5}) and S-acetoacetyl-N-acetylcysteamine (570×10^{-5}) were converted. Pantetheine and pantetheine-4′-phosphate were equally effective as acceptors of the acetyl group instead of CoA (Michaelis

constants, HSCoA: 2.0×10^{-5}; SH-pantetheine: 10.5×10^{-5}; SH-pantetheine-4'-phosphate: 23.5×10^{-5} mole/liter). These experiments led to a modified thiolase test, independent of CoA, whereby the cleavage of S-acetoacetyl-pantetheine (synthesis from pantetheine and diketone) by SH-pantetheine is measured optically at 303 mμ (73).

In an investigation of the distribution of β-ketothiolase in the animal and human organism, Reinwein (4, 46, 71) carried out the determination by coupling the cleavage of S-acetoacetyl-N-acetyl-cysteamine by CoA (reaction 16) with the acetylation of *p*-nitroaniline (reaction 17) by an enzyme fraction of pigeon liver.

$$\text{S-acetoacetyl-N-acetylcysteamine} + \text{CoA} \rightleftharpoons \text{acetyl-CoA} + \text{S-acetyl-N-acetylcysteamine} \qquad 16.$$

$$\text{Acetyl-CoA} + H_2N\text{-}C_6H_4\text{-}NO_2 \rightleftharpoons \text{CoA} + CH_3CONH\text{-}C_6H_4\text{-}NO_2 \qquad 17.$$

A remarkable result of this investigation was the finding that human organs (thiolase content: liver, 100; kidney, 52; heart, 48; brain, 9) are more than twice as active as the same organs of beef, sheep, rabbit, rat or hog (71). The brain contains less enzyme in man and animal in conformity with the slight activity of this organ in the oxidation of fat (72).

The direct determination of β-ketothiolase in extracts of *Cl. kluyveri* (28), *Cl. saccharobutyricum* (61), and *Cl. butyricum* (62) has been reported.

The equilibrium of reaction 11 is on the side of thiolytic cleavage but may be shifted by raising the pH or addition of Mg^{++} (salt or chelate formation) to the condensation side. The equilibrium constant between pH 9 and 7, was found with $K_{eq}' = [\text{acetyl-SCoA}]^2/[\text{acetoacetyl-SCoA}][\text{HSCoA}]$ and gave values of 1×10^4 to 6.4×10^4 (2, 17, 67, 73). The value of pH 7 had to be computed from the equilibrium of the reductive condensation of acetyl-CoA to D-β-hydroxybutyryl-CoA (catalyzed by β-ketothiolase and β-ketoreductase in the presence of DPNH) as the optical method failed to work at the neutral point (73). At pH 7 the equilibrium constant of the combined reaction (equation 10 and 11) was found to be $K_{eq}' = [\text{acetyl-SCoA}]^2(\text{DPNH}]/[\text{D-}\beta\text{-hydroxybutyryl-SCoA}][\text{HSCoA}][\text{DPN}] = 1.2\times10^2$ (25°). Coupling of the condensation with the reduction is of importance for the process *in vivo* since thus the synthesis of fatty acid chains is made possible in spite of the unfavorable condition of the β-ketothiolase equilibrium (59).

From the fact that β-ketothiolase is inhibited by SH-poisons like iodoacetic acid or As_2O_3 Lynen (2) concluded the following to be the mechanism of action of the enzyme (HS—E = enzyme):

$$RCH_2\cdot CO\cdot CH_2\cdot COSCoA + HS\text{—}E \rightleftharpoons RCH_2\cdot COS\text{—}E + CH_3\cdot COSCoA \qquad 18.$$

$$RCH_2COS\text{—}E + HSCoA \rightleftharpoons RCH_2\cdot COSCoA + HS\text{—}E \qquad 19.$$

This scheme was confirmed by experiments with labelled CoA. Lynen (2) showed propionyl-CoA to take up radioactivity on incubation with $HS^{35}CoA$ and purified β-ketothiolase. This is in favour of the reaction:

$$\text{Propionyl—SCoA} + \text{HS—E} \rightleftharpoons \text{Propionyl—S—E} + \text{HSCoA} \qquad 20.$$

An intermediary step is the reversible splitting of β-ketovaleryl-CoA. Ochoa's (69) observation of keeping solutions of purified thiolase in active form by addition of SH-compounds also points to the presence of a functionally important SH group in the enzyme. Considerations of reaction kinetics made Goldman (67) postulate the intermediary formation of an acyl-enzyme compound in the thiolase reaction.

Formation and Consumption of Acetoacetic Acid

Acetoacetic acid is formed in the degradation of normal fatty acids through acetoacetyl-CoA, which originates by oxidation from butyryl-CoA or by condensation of two molecules of acetyl-CoA. Liberation of acetoacetate in the liver takes place by hydrolysis (reaction 21) in the presence of a highly active deacylase (67a, 74, 75) found in liver extracts.

$$\text{Acetoacetyl—CoA} + H_2O \rightarrow \text{Acetoacetate} + \text{CoA} \qquad 21.$$

It appears that a similar enzyme exists in extracts of *Cl. kluyveri* (28, 76). In muscle, acetoacetic acid occurs by reactions 22 and 23, or 22 and 24 respectively. These mechanisms have been discovered simultaneously by Stern *et al.* (67a) and by Green *et al.* (68) in experiments with hog heart extracts.

$$\text{Acetoacetyl—CoA} + \text{Succinate} \rightleftharpoons \text{Acetoacetate} + \text{Succinyl—CoA} \qquad 22.$$

$$\text{Succinyl—CoA} + H_2O \rightleftharpoons \text{Succinate} + \text{CoA} \qquad 23.$$

$$\text{Succinyl—CoA} + \text{ADP} + \text{Pi} \rightleftharpoons \text{Succinate} + \text{CoA} + \text{ATP} \qquad 24.$$

CoA transferase, succinyl-CoA acetoacetate transferase.—Starting with phosphate extracts from hog heart Stern *et al.* (17, 69) achieved a 700- to 1000-fold concentration of the enzyme for reaction 22. The almost homogeneous enzyme fraction catalyzed the reversible CoA transfer from succinyl-CoA to acetoacetate, β-ketovalerate, β-ketoisocaproate and β-ketocaproate. β-Ketooctanoate was inactive. On the other hand, only succinate (not malonate, β-hydroxybutyrate, crotonate, butyrate, or octanoate) proved active as a partner of acetoacetyl-CoA. Beinert & Stansly (77) indicated a much broader specificity based on experiments with crude enzyme fractions. The equilibrium constant of reaction 22 was found to be $K_{eq}' = [\text{succinate}][\text{acetoacetyl-CoA}]/[\text{succinyl-CoA}][\text{acetoacetate}] = 10^{-2}$ (pH 7) (17).

In Ochoa's laboratory a rapid incorporation of C^{14} into succinyl-CoA was observed on incubation of succinyl-CoA with purified CoA transferase and C^{14}-succinate, which suggests intermediary formation of an enzyme-CoA-compound according to the following transferase reaction (E = enzyme):

$$\text{Succinyl-SCoA} + \text{E} \rightleftharpoons \text{Succinate} + \text{E—SCoA} \qquad 25.$$

$$\text{E—SCoA} + \text{Acetoacetate} \rightleftharpoons \text{Acetoacetyl-CoA} + \text{E} \qquad 26.$$

P-Enzyme.—Reaction 23 is catalyzed by succinyl-CoA deacylase (25, 78, 79), reaction 24 by P-enzyme (25, 80) (phosphorylating enzyme). The latter should be of greater importance for the process *in vivo*, since thus a phosphorylation occurs at the substrate level with formation of acetoacetate (67a).

Kaufman *et al.* (25), as well as Hift *et al.* (80), reported on the purification of P-enzyme from heart muscle. Kaufman & Alivisatos (81) isolated the enzyme also from spinach. The equilibrium constant of reaction 24 was found to be $K_{eq}' = [\text{Succinate}][\text{ATP}][\text{CoA}]/[\text{Succinyl-CoA}][\text{ADP}][\text{P}] = 3.3$ (pH 7.4, 20°) (81).

Making use of the purified spinach enzyme, free of myokinase and ITP-ADP transphosphorylase, Kaufman & Alivisatos (81, 82) found incorporation of P^{32}-ADP into ATP and of succinate-C^{14} into succinyl-CoA. They proposed the following series of reactions which only require a single enzyme (Enz = enzyme)

$$\text{Enz} + \text{ATP} \overset{Mg^{++}}{\rightleftharpoons} \text{Enz-Pi} + \text{ADP} \qquad 27.$$

$$\text{Enz-Pi} + \text{CoA} \rightleftharpoons \text{Enz(P—CoA)} \qquad 28.$$

$$\text{Enz (P—CoA)} + \text{succinate} \overset{Mg^{++}}{\rightleftharpoons} \text{Enz} + \text{succinyl-CoA} + \text{Pi} \qquad 29.$$

But, according to Sanadi *et al.* (83 to 86), two enzymes and GDP (or IDP) as coenzyme, participate in reaction 24. One of the two enzymes separated from beef heart mitochondria by Sanadi *et al.* (83), or by Gibson (87) from hog kidney, catalyzes reaction 30 (enzyme A); the other enzyme, reaction 31:

$$\text{Succinyl-CoA} + \text{GDP} + \text{Pi} \overset{Mg^{++}}{\rightleftharpoons} \text{Succinate} + \text{CoA} + \text{GTP} \qquad 30.$$

$$\text{GTP} + \text{ADP} \overset{Mg^{++}}{\rightleftharpoons} \text{GDP} + \text{ATP} \qquad 31.$$

Enzyme A also catalyzes an arsenolysis of succinyl-CoA whereby GDP is not needed (85, 87). The observations of the investigators in New York (67a) and Madison (68) that enzyme fractions of heart muscle combined with CoA are able, only in the presence of catalytic amounts of succinate, to synthesize citrate from acetoacetate, oxaloacetate, and ATP is based upon the cooperation of P-enzyme, CoA transferase, thiolase (reaction 11, 22, 24), and condensing enzyme. In the process *in vivo*, of course, the formation of succinyl-CoA with the oxidation of α-keto-glutarate (24, 25) may have greater importance. In consequence of the interplay of α-ketoglutaric dehydrogenase and CoA transferase there is a direct connection between the citric acid cycle and acetoacetate activation. The P-enzyme bridges the reactions to the phosphate cycle. The close involvement between the citric acid cycle,

phosphorylation, and acetoacetate transformation opens new aspects with regard to the formation of keto compounds in the animal organism.

The presence of CoA transferase and of α-ketoglutaric dehydrogenase in kidney extracts supplies an explanation for the specific effect of α-ketoglutarate which Hunter & Leloir (88) observed in the synthesis of citrate from oxaloacetate and acetoacetate. According to Stern *et al.* (67a) an activation of acetoacetate in the kidney is also achieved by the pyrophosphate cleavage of ATP (reaction 32).

$$\text{Acetoacetate} + \text{CoA} + \text{ATP} \rightleftharpoons \text{Acetoacetyl-CoA} + \text{AMP} + \text{PP} \qquad 32.$$

A similar enzyme is also found in yeast and, in limited concentration, in liver extracts (67a).

Millerd & Bonner (42) found extracts of spinach acetone powder also capable of forming acetoacetate from acetate and ATP. The increase of the synthesis by succinate seems to indicate the cooperation of an enzyme similar to CoA transferase.

Asymmetric Labelling of Acetoacetate

The phenomenon wherein the oxidation of labelled fatty acids by liver slices, liver homogenate, or liver mitochondria produces asymmetrically labelled acetoacetic acid [cf. Kennedy & Lehninger (89)] has been further studied.

Brown & Chaikoff (90) discussed the underlying regularity concerning isotope distribution in acetoacetic acid, founded on the hypothesis of Crandall, Brady & Gurin (91) that the oxidative degradation of fatty acids in metabolism produces two different kinds of C_2 fragments which slowly change into one another: (*a*) one of the ω type (89), stemming exclusively from the two terminal carbon atoms of the fatty acid molecule, which preferentially enter the CH_3CO half of acetoacetic acid, and (*b*) a carboxyl type (89) C_2 fragment from all the other carbon atoms of the fatty acid chain entering specifically into the $—CH_2—COOH$ part of acetoacetate.

Brown *et al.* (90) found theory and experiment to be in close agreement. Another confirmation of this theory (92) was brought about by experiments with oxidation of C^{14}-palmitic acid by liver slices, whereby the acid labelled in position 1, 3, 11, 13, or 15 was injected into rats in the form of the triglyceride. In the experiment with 15-C^{14}-palmitate the *CO/*COOH ratio reached the value 6.0; in all the other experiments the value was near or below 1. (The deviating result of previous similar experiments (93) could not be reproduced.)

The radioactivity of CO_2 formed in the oxidation of labelled fatty acid permitted the calculation that 40 to 50 per cent of C_2 fragments of the ω type acquire identical activity as the C_2 fragments of the carboxyl type in such experiments *in vitro* (90, 92, 94).

Drysdale & Lardy (43) also discovered the formation of asymmetrically labelled acetoacetic acid (*CO/*COOH:0.5–0.7) while studying the oxida-

tion of 1-C^{14}-butyrate and -caproate by soluble enzymes of rat liver mitochondria.

This problem found a simple solution on explanation of the mode of action of the enzyme β-ketothiolase by Lynen (2, 16). According to the mechanism of this enzyme (cf. reaction 18 and 19) the terminal CH_3-CH_2 group of a fatty acid is the only C_2 unit that combines with β-ketothiolase before passing the "acetyl-CoA pool" in the course of oxidation in the fatty acid cycle. This occurs in the last rotation of the cycle when acetoacetyl-CoA from butyryl-CoA is converted by β-ketothiolase into acetyl enzyme and acetyl-CoA (reaction 18). The acetyl enzyme formed in this way, corresponds to the ω type of the C_2 fragment of Crandall *et al.* (91) and has two possibilities of further conversion: either transfer of the acetyl group to CoA (reaction 19), corresponding to the conversion of ω type into carboxyl type C_2 fragments, or condensation with acetyl-CoA from the "pool" with re-formation of acetoacetyl-CoA (reaction 18). If reaction 18 progresses faster than reaction 19 the terminal C_2 fragment of fatty acid will be found chiefly in the carbonyl part and all the rest of the C_2 fragments mainly in the carboxyl part of acetoacetyl-CoA and the resulting acetoacetic acid, respectively.

Beinert & Stansley (77) to whom the functionally active SH group in β-ketothiolase had remained unknown explained the asymmetrical labelling of acetoacetate by the reactions:

$$\text{Enzyme}+CH_3\cdot CO\cdot CH_2\cdot COSCoA \rightleftharpoons \text{enzyme-}CH_3\cdot CO\cdot CH_2\cdot COSCoA \qquad 33.$$

$$\text{Enzyme-}CH_3\cdot CO\cdot CH_2\cdot COSCoA+HSCoA \rightleftharpoons \text{enzyme-}CH_3\cdot COSCoA+CH_3\cdot COSCoA \qquad 34.$$

They assumed also that dissociation of the enzyme-acetyl-CoA complex (enzyme-$CH_3\cdot$ COSCoA) occurs more slowly than the previous thiolysis or condensation step, respectively. The two authors had observed that incubation of acetoacetate and labelled acetyl-CoA with an enzyme fraction from hog heart containing β-ketothiolase and CoA transferase produced asymmetrically labeled acetoacetic (*CO/*COOH <1). The two mechanisms do not differ in principle if in the hypothesis of Beinert *et al.* (77), in lieu of the enzyme-acetyl-CoA complex, is put the acetyl enzyme compound (67) resulting from acyl transfer.

Fatty Acid Degradation in Complete Systems

Clostridium kluyveri.—In a continuation of the investigations on fatty acid oxidation by soluble enzymes from *Cl. kluyveri*, Stadtman (28) was able to prove that degradation of butyrate to acetate or acetylphosphate goes by way of butyryl-CoA and acetoacetyl-CoA. Identification of acetoacetyl-CoA was accomplished by reaction with hydrazine, thus forming 3-methyl-5-pyrazolone. Lieberman & Barker (95) found that the oxidation of valeric or caproic acid by dry cell preparations of *Cl. kluyveri* stops, in the absence of phosphate, at the corresponding β-keto acid. The consumption of β-keto acid in the presence of phosphate could be explained by the intermediate forma-

tion of β-ketoacyl-CoA derivative, its thiolysis by CoA, and regeneration of free CoA from acetyl- or propionyl-CoA respectively via the phosphotransacetylase reaction.

Liver.—Drysdale & Lardy (43) were the first to achieve an oxidative degradation of fatty acids by a soluble system of animal origin. They found that clear extracts of rat liver mitochondria decolorize 2,6-dichlorophenolindophenol in the presence of fatty acids (C_4 to C_{18}) and ATP, if the dye be added to the system successively in proportion with its reduction. Depending upon whether or not oxaloacetate be added to the system, citrate or acetoacetate appears as an oxidation product. After treatment of the mitochondria extract with Dowex 1 the system required CoA and, in the presence of hydroxylamine, hydroxamates of fatty acids were formed—a fact which led to the conclusion that the primary product is a CoA-thioester. 2,4-Dinitrophenol and fluoride had no influence upon the oxidation in soluble systems, although they inhibit oxidation of fatty acids by mitochondria.

A series of investigations was devoted to the oxidation of fatty acids by slices or particle-containing preparations. The process of "priming" in the octanoate oxidation by mitochondria was studied by Judah & Rees (96). With mitochondria from rat liver the oxidation could be induced by ATP; mitochondria from rabbit kidney and liver required malate. The authors explained the divergent behaviour of the kidney by the inferior capacity of this organ to form acetoacetate, so that the regeneration of CoA from acetyl-CoA would be possible only by way of the citric acid cycle.

Oxidation of butyrate in mitochondria may be inhibited by small quantities of 4-fluorobutanoic acid or 6-fluorohexanoic acid ($1.78\times10^{-4}M$). Dominguez *et al.* (97) observed that both acids are converted into the corresponding CoA derivatives by the isolated "activating enzyme" from liver while activation of the fluorine-free acid remains unaffected. They supposed therefore that the fluoro acids inhibit a phase of the fatty acid cycle between the process of activation and the condensation of acetyl-CoA with oxaloacetate.

Geyer *et al.* (98, 99), in experiments with rat liver slices, observed a stimulation of the degradation of octanoate-1-C^{14} by K^+ and Li^+, leading first of all to an increased formation of acetoacetic acid. The ratio $CH_3\cdot CO\cdot CH_2\cdot C^{14}OOH/C^{14}O_2$ which was below 1 in a medium containing only Na^+ rose in the presence of K^+ and Li^+ to values over 3 (99). In the degradation of the odd numbered valeric acid-1-C^{14} K^+ again was stimulatory but the increase concerned almost equally the formation of acetoacetate and of CO_2. Geyer *et al.* (100) assume a normal intracellular K^+ level in the tissue slices to be necessary for a high fatty acid metabolism, which is assured only by a high potassium concentration in the medium.

Lyon & Geyer (101), using respiring slices of rat liver and octanoate-1-C^{14}, studied C^{14} incorporation into CO_2, acetoacetate, fatty acids of various chain lengths, and cholesterol. They found that an increased incorporation into higher fatty acids could be obtained by addition of pyruvate,

succinate, or fumarate with a simultaneous reduction in the formation of acetoacetate. Malonate possessed the opposite activity, namely, stimulation of synthesis of acetoacetate and short chain fatty acids as well as inhibition of CO_2 formation. The C_2 fragments from the experiments with malonate could be reconverted by pyruvate, succinate, or fumarate into higher fatty acids. The C^{14} incorporation into higher fatty acids was promoted by high K^+ concentrations.

Artom (102, 103) discovered an increase in the $C^{14}O_2$ formation from stearate- or palmitate-1-C^{14} in liver slices or liver homogenates when choline-deficient rats were injected with choline approximately one hour before being sacrificed. He referred this to an acceleration of the fat oxidation by substances—possibly phosphatides synthesized *in vivo* from choline. A similar effect was observed recently in homogenates of kidney, heart, and brain, but not of testes (104). The fact, known from experiments *in vivo*, that even-numbered higher fatty acids yield more acetoacetic acid on degradation than odd-numbered ones has been confirmed by experiments with washed liver mitochondria. Witter *et al.* (105) found that C_{2n} fatty acids (C_4 to C_{16}) were quantitatively oxidized to acetoacetate while the C_{2n-1} acids yielded only a little more than 1 mole of acetoacetate per mole of acid oxidized.

Scholefield *et al.* (106, 107) demonstrated that the degradation of carboxyl-labelled alkylthio fatty acids by liver follows the pattern of β-oxidation. It was possible to identify mercaptan, which, in the case of ethylthiopropionate, was formed through ethylthiolcarbonate, while the path of oxidation of alklythioacetic acid remained obscure. Ethylthioacetate, added to liver slices, caused inhibition of $C^{14}O_2$ formation from α-C^{14}-pyruvate as well as of acetoacetate formation from acetate or butyrate, which the authors attribute to the formation of ethylthioacetyl-CoA from CoA. Thus, the inhibition in the formation of acetoacetic acid by ethylthioacetate would be similar to that by propionic (108) or benzoic acid (107). It would be conceivable that the inhibition of fatty acid oxidation by 2-(2-naphthydroxy-) propionic acid and 2-(6-bromonaphthydroxy-) propionic acid (109) is of similar origin.

It is known that damage of mitochondria injures their capacity to oxidize fatty acids. According to Christie & Judah (110) it is such a disintegration of liver mitochondria that underlies CCl_4 poisoning. Lévy & Legrand (111) attribute the damage done by "toxic filtrate" of *Cl. welchii* to octanoate oxidation in rat liver homogenates to the cleavage of mitochondria phosphatides by lecithinase present in such filtrates.

Heart.—Crotonate, vinylacetate, β-hydroxybutyrate, and acetoacetate were identified as products of the oxidation of β-C^{14}-butyrate in rabbit ventricle slices (114, 115). Crotonate was roughly 10 times more active than vinylacetate and contained C^{14} mainly in the β-position (115) while β-hydroxybutyrate and acetoacetate showed high radioactivity in the carboxyl group as well (114). Lactate, pyruvate, or fumarate contained C^{14} only in the carboxyl position, in harmony with their origin from butyrate through the citric acid cycle.

Kidney.—Lang & Bässler (112) found that cell particles from rabbit kidney, but not from liver, heart, brain, lung, or skeletal muscle (113), were capable of degrading homologous dicarboxylic acids from glutaric to sebacic acid as well as itaconic acid. Intermediate products were not found.

Plants.—Fawcett, Ingram, & Wain (116) studied the degradation of homologous ω-phenoxyalkylcarboxylic acids [$C_6H_5O(CH_2)_nCOOH$ with n = 1 to 10] in flaxseed and found, as was to be expected on the basis of a β-oxidation, considerable amounts of phenol to be formed only from acids in which n was an even number. The acid of n = 9 reacted abnormally, also leading to the formation of phenol; this was attributed to an ω oxidation. Observations on the growth-promoting capacity of homologous ω-(4-chlorophenoxy)-, ω-(2,4-dichlorophenoxy)-, and ω-(2,4,5-trichlorophenoxy)-alkylcarboxylic acids or of homologous ω-(1-naphthyl)-alkylcarboxylic acids agree likewise with the theory of β-oxidation (117), as Synerholm & Zimmerman (118) have already pointed out. It is remarkable that the plant material investigated by Wain & Wightman (117), wheat coleoptile, pea stalk and tomato leaf, showed different oxidative capacities. Thus, e.g., the conversion of ω-(2,4,5-trichlorophenoxy)-butyric acid to the corresponding acetic acid derivative, active as growth promoter, could only be detected in the wheat coleoptile. Alkylation in the β-position of the side chain prevents β-oxidation.

Newcomb & Stumpf (119) investigated the synthesis and degradation of fatty acids by peanut cotyledons. Slices of cotyledons were able to incorporate C^{14}-acetate in higher fatty acids (more than 10 carbon atoms) under aerobic as well as anaerobic conditions; dinitrophenol, 10^{-3} *M*, inhibited this process completely. Study of $C^{14}O_2$ formation from 1-C^{14}-palmitate with extracts of germinating cotyledons revealed two active fractions: (*a*) microsome-like particles sedimenting at 100,000 g and (*b*) soluble cytoplasmic proteins. The protein mixture which could be precipitated from fraction *b* by ammonium sulfate required a cofactor (= CoP), present in the supernatant, which was stable to boiling with 0.1 *M* alkali or 0.1 *M* acid, the purification of which was taken up by Castelfranco *et al.* (120). CoP possesses the qualities of an acid and is identical with none of the known coenzymes. It was discovered in peanut cotyledons as well as in mammalian liver, *Cl. kluyveri*, wheat germ, spinach leaves, and yeast. The oxidation system from the microsomes, thoroughly investigated by Humphreys *et al.* (121), is also different from the "fatty acid oxidase" of animal tissues or microorganisms. It was found, e.g., that in this system (*a*) palmityl-CoA is inactive, (*b*) only DPN is required as cofactor, (*c*) 2-C^{14}-palmitate yields more $C^{14}O_2$ than 3-C^{14}-palmitate, and (*d*) the citric acid cycle does not participate in the CO_2 formation. The chemical nature of the reaction products other than CO_2 is not established. Newcomb & Stumpf (119) propose the following reaction sequence and report that they have been able to identify labelled n-tridecylmethylketone in the degradation of 3-C^{14}-palmitate.

$$R \cdot CH_2 \cdot CH_2 \cdot COOH \rightarrow R \cdot CH{:}CH \cdot COOH \rightarrow R \cdot CO \cdot CH_2 \cdot COOH \rightarrow R \cdot CO \cdot CH_3 + CO_2$$

The upper limit of activity of the microsome enzyme rests with lauric acid, with 12 carbon atoms (121).

By making use of Bindschedler's Green as hydrogen acceptor Franke & Frehse (122) could identify dehydrogenases for higher fatty acids in various plant seeds. While beets, rape, and pumpkin were active only with higher saturated fatty acids (down to C_{10}), cereal grains, soybeans, linseed, hemp, and sunflower dehydrogenated linolic- and linolenic acid as well. The authors attribute this to the presence of two different enzymes. The enzymes were active also after dialysis and appeared not to need the addition of coenzymes. Since identification of the reaction products is still missing the biological importance of these enzymes is yet to be evaluated. From the fact that preliminary lipoxidatic oxidation of linolic or linolenic acid accelerated the subsequent dehydrogenation by seed extracts, Franke *et al.* concluded that dehydrogenase and lipoxidase collaborate in the degradation.

Fatty Acid Synthesis

Cell-free systems.—Synthesis of carbon chains from acetyl-CoA in the fatty acid cycle is dependent on the presence of DPNH and the availability of reducing agents for the flavin enzymes. With this in mind, Stansly & Beinert (123) built up a system of purified enzymes in which $CH_3{}^{14}COSCoA$ was converted with a yield of 15 per cent into radioactive butyryl-CoA but not into higher acyl CoA's.

Synthesis of long chain fatty acids from C^{14}-acetate has been achieved thus far only by utilizing crude extracts of liver tissue or of lactating mammary glands. The stimulating activity of citrate, observed by Dituri & Gurin (124) in experiments with rat liver mitochondria and extracts thereof, may partially be associated with the production of ATP and DPNH. Van Baalen & Gurin (125) discovered that the incorporation of C^{14}-acetate into higher fatty acids in pigeon-liver extracts treated with charcoal is dependent on the addition of ATP, DPN and CoA.

The enzyme system of mammary glands was thoroughly investigated by Popják & Tietz (126) by means of C^{14}-acetate. Sheep udder, without any further addition, is capable of degradation of acetate and synthesis of fatty acids while both processes occur in the mammary gland of the rat only in the presence of glucose or of acids of the citric acid cycle. The synthesis of fatty acids, dependent on aerobic conditions and ATP, was considerably increased in homogenates by addition of oxaloacetate. C^{14}-acetate was incorporated by all even-numbered fatty acids, but the main quantity went to caproic acid (24 per cent), myristic acid (13.6 per cent), palmitic acid (35.6 per cent), and oleic acid (17.6 per cent). On fractionation of homogenates of rat mammary gland the activity for fatty acid synthesis remained in the supernatant fluid obtained after removal of mitochondria and microsomes (127).

Intact cells.—That the sequence, glucose→pyruvate→acetate→fatty acids, occurs also in the synthesis of milk lipides in the rabbit was shown by

Popják, Hunter & French (128) in experiments with C^{14}-labelled acetate, pyruvate, or glucose. The glycerol in the fat derived from glucose was converted more quickly than the acid components.

Capacity for the synthesis of fat from acetate is greater in the mammary glands of sheep than of rats (126, 129). Balmain, Folley & Glascock (129), with a triple-labelling technique found, in the investigation of lipogenesis from $CH^3{}_3$-$C^{13}OOH$ or C^{14}-glucose, that acetate carbon and glucose carbon are utilized, respectively, in the proportion 38:62 by rat-tissue slices, and 97:3 by sheep-tissue slices. Insulin raised the utilization of both substrates to the same extent; glycerol favored only the incorporation of acetate.

Production of fat during lactation is attended by a considerable increase in the CoA content of the mammary gland. Ringler *et al.* (130) found in guinea pig glands during pregnancy 20 to 30 units of CoA/gm. tissue, but five days after birth a transitory maximum of 170 CoA units. In contrast, the DPN content increased only slightly.

According to a calculation by Richardson & Folley (131) an epithelial cell of goat udder synthesizes 4.1×10^6 molecules of fat (corresponding to 21.5×10^6 condensations), 4.5×10^6 molecules of lactose, and 1.5×10^{-9} μg. casein in one second.

Kleiber *et al.* (132) determined the radioactivity of respiratory CO_2 and organic constituents of milk from cows injected with 1- or 2-C^{14}-butyrate and found more C^{14} in lactose and casein than in milk fat. The authors concluded that there was a conversion of butyrate to lactose, and avoidance of the "acetate pool." Feller (133) studied the incorporation of C^{14}-acetate into various lipide fractions by adipose tissue slices of rat. The unsaponifiable matter (mainly cholesterol) took up scarcely any C^{14} though the fatty acid fraction did so, especially after addition of succinate or glucose. In contrast to liver where both acetate carbon atoms are incorporated at the same rate in fatty acids, C^{14}-incorporation of 1-C^{14}-acetate is twice as fast as of 2-C^{14}-acetate in adipose tissue. For this reason Feller concludes that the separate incorporation of both carbon atoms through a C_1 compound other than CO_2 is possible.

Regeneration of CoA and Synthesis of the Ester Linkage

Liberation of CoA from the higher acyl CoA compounds formed in the course of synthesis may occur, in principle, by hydrolysis (equation 35) or by acyl transfer to acceptors like glycerol, α-glycerophosphoric acid and similar compounds (equation 36).

$$\text{RCO—SCoA} + \text{H}_2\text{O} \rightarrow \text{R—COOH} + \text{HSCoA} \qquad 35.$$

$$\text{RCO—SCoA} + \text{HO—R}' \rightarrow \text{RCO—O—R}' + \text{HSCoA} \qquad 36.$$

Acyl transfer would be much more reasonable since thus the energy of the acyl mercaptan bond could be used directly for syntheses. The observation made by Balmain *et al.* (134) that fatty acid synthesis from acetate by rat mammary gland slices is increased considerably by glycerol points in this

direction. On the other hand, Van Baalen & Gurin (125) find in the fatty acid fraction a higher radioactivity than in the glyceride fraction in their investigation of lipogenesis from C^{14}-acetate by pigeon liver extracts; this indicates the possibility of a hydrolytic cleavage.

Deacylases, thioesterases.—Deacylases for long chain acyl CoA compounds have not been identified so far. Unequivocal proof is limited to specific deacylases for acetyl-CoA (79), succinyl-CoA (25, 78, 79) and acetoacetyl-CoA (67a, 74). Besides these, deacylases were described for S-acyl- (136, 137) and S-aminoacyl-glutathione derivatives (138a), as well as for nitrogen-free simple thioesters of the type of S-methyl-, and S-ethyl-thioacetate and others (139, 140, 141). Hydrolysis of the latter, which occurs in liver tissue with considerable speed, is attributed to the ordinary liver esterase (140, 141).

Acyl transfer to glycerol derivatives.—Kornberg & Pricer (9) detected in guinea pig liver an enzyme which synthesizes phosphatidic acids from acyl CoA compounds and α-glycerolphosphate according to equation 37. Experiments of Dawson (142, 143) proved an identical enzyme to exist in brain.

$$2\ \mathrm{RCO{-}SCoA} + \begin{matrix}\mathrm{H_2COH}\\ |\\ \mathrm{HCOH}\\ |\\ \mathrm{H_2COPO_3H_2}\end{matrix} \rightarrow 2\mathrm{HSCoA} + \begin{matrix}\mathrm{H_2COCOR}\\ |\\ \mathrm{HCOCOR}\\ |\\ \mathrm{H_2COPO_3H_2}\end{matrix} \qquad 37.$$

This enzyme which remained in the sediment in the fractionation of liver homogenate has a distinct activity optimum with CoA derivatives of C_{16} to C_{18} acids. The preferential occurrence of palmityl- and stearyl glycerides in the animal body might be thus explained (59). The enzyme fraction did not react with free glycerol or with β-glycerophosphate.

The function of the phosphatidic acids as intermediate products in the synthesis of phosphatides has been elucidated by Kennedy (144, 145). It emerges that lecithin synthesis by rat liver mitochondria, linked to the presence of ATP, leads to α-glycerophosphoric acid as an intermediate, the synthesis not taking place through phosphorylcholine or L-α-glycerylphosphorylcholine; this was studied by means of radioactive phosphate (144) and choline (145). In continuation of these experiments Kennedy (146) discovered, furthermore, that extracts of lyophylised mitochondria can combine dipalmitoyl-L-α-glycerophosphate with choline to yield lecithin if ATP, CoA, and cysteine are present. Preliminary experiments suggest CoA to be necessary for the activation of phosphatidic acid (analogous to carboxylic acid activation). It should be mentioned that Dawson (142) was unable to demonstrate the presence of phosphatidic acids in liver and brain; their concentration *in vivo* must therefore be very low. This does not, however, concern in principle the function of phosphatidic acids as intermediate products.

Jedeikin & Weinhouse (147) tested various rat tissues for their capacity to incorporate 1-C^{14}-palmitate in lipides and found the highest activity to be in kidney, heart, spleen, and liver. While intact respiration and the produc-

tion of ATP were required for phosphatide synthesis, incorporation of the labelled fatty acid residue into the phosphorus-free lipide fraction also took place in the presence of respiratory and phosphorylating poisons. This led Jedeikin *et al.* to conclude that the ester linkages of phosphatides are built up with the participation of CoA thioesters, the synthesis of glyceride-ester bonds, however, probably proceeding in another way. In this connection it is noteworthy that lipase catalyses the synthesis of triglycerides from diglyceride and fatty acid (148). But it is to be questioned whether this reaction, although being of importance in the process of digestion in the small intestine has any significance in the synthesis of fat in the organs. In general lipase is absent from organs.

It also has not yet been clarified whether interchange of ester linkages, which has been discovered recently in studies of esterase, participates in the synthesis of fat. Handler & Stewart (149) found incubation of horse-liver esterase with ethyl butyrate, butyrate, and 1-C^{14}-ethanol to cause C^{14} uptake with partial hydrolysis of the ester, which seems to support the following mechanism of enzyme action:

$$\text{Ethyl butyrate} + \text{enzyme} \rightleftarrows \text{enzyme butyrate} + \text{ethanol} \qquad 38.$$

$$\text{Enzyme butyrate} + H_2O \rightarrow \text{enzyme} + \text{butyrate} \qquad 39.$$

In this connection the process of enzymatic thioltransacetylation, discovered by Brady & Stadtman (150) in *Cl. kluyveri* and pigeon liver, seems noteworthy. It appears to be promising to search for analogous enzymes which would catalyse acyl transfer between O-ester and alcohol.

A difference in the pathways which lead to phosphatides and triglycerides seems probable also from observations of Gidez & Karnovsky (151) in experiments with rats. After incorporation of C^{14}-glycerol in the triglyceride fraction of liver they found the specific activity thereof to be threefold that of the phosphatide fraction. In the evaluation of these experiments heterogeneity of the phosphatide fraction has to be considered, of course.

In the opinion of the reviewer it is therefore still to be taken into account that phosphatidic acids participate in the biosynthesis of fats, be it as sources of the diglyceride unit or as acyl donor in transacylation reactions.

Lipogenesis

With the insight now gained into the process of lipogenesis the close relationship to carbohydrate metabolism is understood more readily. It is the oxidation of sugar which, besides ATP and acetyl-CoA, provides the hydrogen equivalents in the form of DPNH and $FADH_2$ necessary for the synthesis of fatty acid. In addition, sugar is the mother substance of phosphorylated or free glycerol needed in the synthesis of phosphatides and fat, and the availability of it, in connection with the uptake of higher fatty acid residues from CoA, might possibly even limit the rate of lipogenesis.

The reduction in lipogenesis in the hungry or diabetic organism has been further investigated. Hutchens *et al.* (152) confirmed in the rat inhibition of

lipogenesis in prolonged fasting or in alloxan diabetes (153). Damage through fasting was more evident in the liver than in intestines, carcass, or skin. Strangely enough, the fat content of the liver remained practically unaltered during a fasting period of up to ten days [cf. also Harrison (154)].

To calculate exactly the rate of conversion of acetate into fat from isotope experiments the specific activity of the "acetyl pool" at the site of lipogenesis must be known. As Medes, Spirtes & Weinhouse (155) showed, it is possible to determine this activity by trapping acetyl groups in the form of acetoacetate. New measurements on liver slices from fasting rats confirm the strong inhibition of lipogenesis with unimpaired acetate oxidation. On the other hand, livers of rats fed richly with carbohydrate regenerate each hour 0.1 to 0.7 per cent of the total fatty acids.

Hausberger *et al.* (156, 157) showed that slices of adipose tissue from alloxan-diabetic rats, as well as liver slices, which had completely lost the capacity to form fat from C^{14}-glucose reacquire this capacity by treatment *in vivo* with insulin. The existence of a common pathway through acetoacetyl-CoA is indicated by the fact that 3-C^{14}-acetoacetate behaves like C^{14}-acetate (158) with respect to incorporation into higher fatty acids by liver slices of normal, hungry, or alloxan-diabetic rats, with or without insulin. If rats are kept at 0 to 2° for some time effects identical with those of hunger occur. In liver slices from such animals conversion of 1-C^{14}-acetate into fatty acids is much reduced in comparison with controls (159). $C^{14}O_2$ formation and incorporation into cholesterol are unaltered. In liver homogenates from rats which have been heavily fed with carbohydrate for some time, incorporation of 2-C^{14}-acetate into fatty acids occurs faster than from animals fed on much fat (160). In respect to incorporation into cholesterol the effects are opposite.

The importance of active glycolysis for lipogenesis was demonstrated by Shaw & Gurin (161) in experiments with liver extracts of alloxan-diabetic rats. In such extracts incorporation of C^{14}-acetate or -pyruvate proceeded only after addition of glycogen, fructose, glucose-6-phosphate, or hexosediphosphate, all of which are able to serve as substrates for glycolysis; glucose proved to be inactive, which would be consistent with the theory of an initial block in glucose utilization in diabetes.

As a consequence of hunger or of alloxan diabetes the ratio DPNH:DPN which is normally 1:2.6 [according to Spirtes & Eichel (162) 1:1.52] falls to 1:4.5 in rat liver, but returns to normal after addition of fructose or of glucose and insulin (163). According to Helmreich *et al.* (163), the DPN-deficiency, which would explain the reduction in lipogenesis in hungry and diabetic rats, is attributable to the interruption of glycolysis.

An argument against a firm coupling of active glycolysis and lipogenesis is a finding of Spirtes, Medes & Weinhouse (164) according to which liver slices of rats treated with thyroxin incorporated C^{14}-acetate into fatty acids at normal rates in spite of low glycogen content. One will have to wait for further experiments in order to explain these contradictory results.

Whether increase of lipogenesis by insulin represents a specific effect or

is the consequence of insulin action upon glucose conversion cannot yet be answered. It was concluded from a series of observations that insulin has not only an effect upon consumption of glucose. Beatty & West (165) assume an insulin effect on the level of the citric acid cycle as well, since, in normal rats, ketonuria may be reduced by succinate alone, while the same effect is achieved in diabetic animals only by succinate and insulin simultaneously. Frohman & Orten (166) found in experiments with C^{14}-acetate that the C^{14}-content of citric acid cycle intermediates in the liver of alloxan-diabetic rats was low in comparison with normals. They consider that this supports the concept of a metabolic block in such animals at the stage of citrate formation. This hypothesis is hard to reconcile with the finding that $C^{14}O_2$ formation from labelled acetate occurs at a normal rate in the liver of the diabetic animal. The same objection may be made against the hypothesis of Ciaranfi & Fonnesu (167, 168) according to which the mechanism for acetyl-CoA synthesis from acetate would be damaged, amongst others, in the diabetic animal. This hypothesis rests on the observation that in pancreatectomized dogs the blood acetate level, after intracardiac injection of acetate, reached a higher value than in the normal animal; it also dropped more slowly.

Inhibition of lipogenesis from acetate by the hyperglycemic-glycogenolytic factor (HGF) was observed by Haugaard *et al.* (169). In a continuation of these experiments on liver slices it was found (170) that HGF also impairs the incorporation of C^{14}-glucose or C^{14}-fructose into fatty acids while simultaneously stimulating keto body formation in the absence of substrates. This shows that HGF, as an over-all effect, promotes oxidation and inhibits synthesis of fatty acids.

Bernhard & Wagner (171) found in experiments on rat liver slices that phytol feeding led to an increase of oxidation of 1-C^{14}-acetate and to a decreased incorporation into higher fatty acids. Absence of fat synthesis in rats on pyridoxin-free diets precedes disturbances in N-metabolism and, according to Beaton *et al.* (172), may be attributed to a defect in energy metabolism consequent to which the capacity for storage of excess food as fat is lost. Incorporation of C^{14}-glucose into body lipides took place at the same rate in B_6-deficient rats as in control animals (173).

Experiments on rats by Ling & Chow (174) indicate that carbohydrate- and fat-metabolism disorders observed in vitamin-B_{12} deficiency may be explained by a lowering of the glutathione content of the blood. Stimulation of fat combustion by growth hormone was confirmed once more by experiments with living rats and with isolated tissues (175, 176); this is accompanied by ketonemia and an increase in activity of fatty acid oxidase which can be observed directly in liver homogenates 12 to 14 hr. after injection of the hormone (177).

Incorporation of fed 1-C^{14}-acetate by liver and body fat of mice with hereditary "obese-hyperglycemic syndrome" is substantially raised as compared with the normal (178). X-irradiation does not influence the combustion of 1-C^{14}-acetate to $C^{14}O_2$ in the rat; incorporation of acetate in fatty acids

of the liver is increased, but is lowered in the fatty acids of the digestive tract. Coniglio & Hudson (179) consider this as an indication of the partial metabolic independence of the various fatty acids of the body.

Metabolism of Propionic Acid and Branched Chain Fatty Acids

The synthesis of normal even-numbered, normal odd-numbered, and branched chain fatty acids is dependent on the successive addition of acetyl-CoA, to acetyl-CoA, propionyl-CoA, or a branched acyl derivative as e.g., α- or β-methylbutyryl-CoA, respectively, as the starting unit. Since in the paunch of ruminants propionic acid, isobutyric acid, and isovaleric acid as well as 2-methylbutyric acid appear in considerable amounts as products of microbial degradation of carbohydrates and protein (cf. 180, 181, 182) and these acids are reabsorbed and utilized by the rumen epithelium (181, 183), it is apparent that odd-numbered as well as branched chain fatty acids are regular constituents of certain animal fats (184 to 189).

Propionic acid.—Degradation of this acid which is finally formed by the successive β-oxidation of odd-numbered fatty acids is still under discussion. There are two contrasting theories: a direct carboxylation of propionate to succinate, and a series of reactions which lead by way of acrylate, L- and D-lactate to pyruvate. The second theory stems from the work of Huennekens *et al.* (190) and is based upon the observation that C^{14} from 1-C^{14}-propionate passes into pyruvate, D- and L-lactate (191), and that mitochondria are only able to oxidize propionate after addition of a soluble cytoplasmic fraction which contains racemase, an enzyme that converts L-lactate to D-lactate (190). Contrary to this is the finding of Lang & Bässler (192) that mitochondria alone are capable of propionate oxidation if an excess of succinate is added as "sparker." Furthermore, the route through acrylate fails to explain the complete "randomization" of the carbon atoms in position 2 or 3 of the propionate which takes place in the synthesis of glycogen (193), lactic acid (194), or acetate (195). The assumption by Mahler *et al.* (191) of enzymatic racemization of lactic acid by way of a symmetric cyclopropane derivative appears improbable from a chemical point of view.

This "randomization" is understandable if degradation of propionate takes place by way of symmetrical succinate. Besides, Lardy (196, 197) was able to demonstrate that extracts of acetone dried rat liver mitochondria catalyze the condensation of $C^{14}O_2$ and propionate to radioactive succinate in the presence of ATP and Mg^{++} or Mn^{++}. Pennington (183) found a considerable stimulation of propionate degradation in the epithelium of sheep rumen if the atmosphere contained 20 per cent CO_2, while the consumption of acetate or butyrate remained unaffected. The author assumes that CO_2 fixation takes place at the level of propionyl-CoA so that this remains when CO_2 is lacking. Hence, if there is insufficient free CoA available for the degradation of endogenous substrates a respiratory inhibition sets in [cf. also Lang & Bässler (192)].

In experiments with cell-free extracts of *Micrococcus lactilyticus* Whiteley (198) found that decarboxylation of succinate required catalytic amounts of

CoA and ATP. The process may be described by equations 40 and 41:

$$\text{Succinyl-CoA} \rightarrow CO_2 + \text{propionyl-CoA} \qquad 40.$$

$$\text{Propionyl-CoA} + \text{succinate} \rightarrow \text{succinyl-CoA} + \text{propionate} \qquad 41.$$

ATP sparks the reaction by initial synthesis of succinyl-CoA from succinate and CoA (reaction 24).

ATP and CoA prove also to be necessary in the decarboxylation of succinate by the cellular fluid, treated with Dowex-1, from *Propionibacterium pentosaceum* (199). In an extension of these experiments Delwiche, Phares & Carson (200) found that extracts of *P. pentosaceum* catalyze a fast incorporation of 2-C^{14}-propionate into succinate at pH 5.6, but acquire the capacity for decarboxylation of succinate, or for incorporation of $C^{14}O_2$ in succinate, only after addition of an enzyme fraction from *Veillonella gazogenes*, which possesses in itself none of the activities mentioned. These and other findings may be explained by reactions 42 (*P. pentosaceum* extract) and 43 (*V. gaz.* extract), the intermediary synthesis of a C_1-compound and activation of succinate by CoA-transphorase (reaction 41).

$$\text{Succinyl-CoA} \rightleftharpoons \text{Propionyl-CoA} + \text{“}C_1\text{”} \qquad 42.$$

$$\text{“}C_1\text{”} \rightleftharpoons CO_2 \qquad 43.$$

Biotin has been observed to participate in the reversible conversion of propionate to succinate. Thus, Lardy *et al.* (197) found that carboxylation of propionate occurs only at 10 per cent of the normal rate in mitochondria from biotin-deficient rats. Chambers & Delwiche (201) observed biotin to stimulate decarboxylation of succinate by cells of *Propionibacterium pentosaceum* cultivated in biotin-free media. Since biotin evidently influences neither the CoA content of the cells nor their faculty for synthesis of succinyl-CoA the authors conclude that biotin participates in the synthesis of an apoenzyme of the decarboxylating system or in the formation of a coenzyme (active C_1-body?). A third possibility would be an unspecific influence of biotin upon protein synthesis in general.

Branched-chain fatty acids.—In continuation of the investigations on degradation of isovaleric acid in the animal body, by which acetoacetate and a C_2 fragment are formed with fixation of CO_2 (237), Bachhawat, Robinson & Coon (203) were able to establish reactions 44 to 47 by experiments with liver and heart extracts.

$$(CH_3)_2CH \cdot CH_2 \cdot COSCoA \rightleftharpoons (CH_3)_2C{:}CH \cdot COSCoA + 2H \qquad 44.$$

$$(CH_3)_2C{:}CH \cdot COSCoA + H_2O \rightleftharpoons (CH_3)_2C(OH) \cdot CH_2 \cdot COSCoA \qquad 45.$$

$$(CH_3)_2C(OH) \cdot CH_2 \cdot COSCoA + CO_2 \overset{(ATP)}{\rightleftharpoons} CH_3 \cdot C(OH)(CH_2 \cdot COOH) \cdot CH_2 \cdot COSCoA \qquad 46.$$

$$CH_3 \cdot C(OH)(CH_2 \cdot COOH) \cdot CH_2 \cdot COSCoA \rightleftharpoons CH_3 \cdot CO \cdot CH_2 \cdot COOH + CH_3 \cdot COSCoA \qquad 47.$$

In the first phase (activation, reactions 44 and 45) degradation of isovaleric acid corresponds to the degradation of normal fatty acids, for it could be demonstrated that the same enzymes [ethylene reductase (53), crotonase (11, 13)] are active therein. Fixation of CO_2 in reaction 46 needs the presence of ATP. It could be concluded from experiments with heart-muscle extracts, freed of crotonase by a short heating, that carboxylation does not occur at the level of β-methylcrotonyl-CoA. Such extracts converted only β-hydroxy isovaleryl-CoA into acetoacetate with fixation of CO_2. Formulation of reaction 47 is based upon the finding with heart muscle extract that acetoacetate and acetyl-CoA originate in about equimolar quantities from synthetic β-hydroxy-β-methylglutaryl-CoA. This cleavage requires the presence of cysteine or glutathione. Application of an optical enzyme test by which cleavage of β-hydroxy-β-methylglutaryl-CoA (reaction 47) is coupled with acetylation of *p*-nitroaniline (reaction 17, enzyme fraction of pigeon liver) permitted a study of the enzyme distribution in the rat (14). Relative specific activities of the organ extracts were: liver, 100; kidney, 67; heart, 20; testes, 6.

From reactions 44 to 47 the stoichiometric incorporation of $C^{14}O_2$ into acetoacetate, which accompanies the biological degradation of isovalerate by liver slices (237) or extracts from liver mitochondria (43), is now comprehensible and also explains the stimulation of the degradation of isovalerate by CO_2 in the epithelium of sheep rumen (181). According to observations in Lardy's laboratory (197), biotin is also concerned in this series of CO_2-fixation reactions. It was found that liver mitochondria of biotin-deficient rats are unable to oxidize isovalerate or β-methylcrotonate with formation of acetoacetate, though oxidation of octanoate occurred at a normal rate. The metabolic defect conditioned by biotin deficiency can be due, therefore, only to reaction 47, or more probably, to reaction 46.

The question about the reversibility of reactions 46 and 47 touches upon a problem of great importance in connection with the biosynthesis of steroids, terpenes, rubber, and so forth. Since Bonner & Arreguin (204) postulated β-methylcrotonic acid as an intermediate product of rubber synthesis in isolated "guayule" leaves, the view that synthesis of natural substances consisting of isoprene units (205) takes place through branched chain fatty acids has become more and more accepted. Experimental data concerning the origin of single carbon atoms of cholesterol from acetate-methyl or acetate-carboxyl, (cf. 206) have been extended by more recent investigations of Cornforth *et al.* (207, 208, 209) and Dauben *et al.* (210). They leave scarcely a doubt that a C_5 body is formed from acetate and, standing in close relationship to isovaleric or β-methylcrotonic acid, is an intermediate product in this synthesis. This hypothesis permitted Cornforth & Popják (211) to explain the distribution of C^{14}-acetate in squalene as built up by rat liver slices. The hypothesis is also in accord with findings on β-carotene from *Mucor hiemales*. Grob & Bütler (212) found the lateral methyl groups of carotene to be formed from the methyl groups of labelled acetate, the vicinal quaternary carbon

atoms of the aliphatic chain, on the contrary, from the carboxyl group. Zalokars' (213) observation that β-methylcrotonic acid does not increase carotenoid synthesis in *Neurospora crassa* is not to be considered as a contrary argument: rate of pigment formation in the fungus may be independent of the supply of exogenous precursors.

The enzyme system of liver which is capable of synthesizing cholesterol has been found able to synthesize β-methylcrotonic acid from acetate. Recent work on this subject is introduced by the work of Bucher (214) which describes a cell-free preparation from rat liver which can synthesize cholesterol from acetate in the presence of ATP and DPN. This cell-free synthesis takes place during several hours at constant rate after a short "lag phase" (215) and responds to CoA as well if high acetate concentrations are used (216). Bucher *et al.* (217) found, in fractionating rat liver homogenate, that the enzymatic activity is distributed between microsomes and supernatant fluid. In experiments by Rabinowitz & Gurin (218), however, only the combination of supernatant with intact mitochondria or with an aqueous extract of mitochondria proved to be active. The second completely water-soluble system contained as effective cofactors: DPN, CoA, and ATP which could be isolated by treatment with activated charcoal. Participation of CoA in steroid synthesis has been demonstrated also by experiments of Klein & Lipmann (219, 220).

The further study of cholesterol synthesis in soluble liver systems showed (218) 1-C^{14}-acetyl-CoA or 2-C^{14}-pyruvate to be more active precursors than 1-C^{14}-acetate; incorporation of acetate was lowered to less than half by addition of β-methylcrotonate or β-hydroxy-β-methylglutarate. Utilization of these C_5 or C_6 acids in cholesterol synthesis follows also from experiments by Bloch *et al.* (221, 222). The authors found, after feeding 3-C^{14}-β-methylcrotonic acid or 3-C^{14}-β-hydroxy-β-methylglutaric acid to rats, that the C^{14} entered liver cholesterol preferentially and was distributed equally in the different parts of the molecule. From this, Bloch concluded that several identical isoprene-like units condense in the synthesis of the steroid skeleton. It is not known if, in this process, the "head-tail condensation" postulated by Lipmann (223) acts between several β-methylcrotonyl-CoA compounds.

Conversion of C^{14}-acetate into β-hydroxy-β-methylglutarate was observed in rat liver homogenates (224) as also in the soluble enzyme system (218) derived therefrom. By chemical degradation of the C_6-acid Rudney (224) was able to define the origin of the carbon atoms and thus to prove the reaction series 48 which has been discussed by various authors (4, 206, 223, 225).

$$2CH_3 \cdot COOH \rightarrow CH_3 \cdot CO \cdot CH_2 \cdot COOH \xrightarrow{+CH_3 \cdot COOH} CH_3 \cdot C(OH)(CH_2 \cdot COOH) \cdot CH_2 \cdot COOH \qquad 48.$$

In this connection should be mentioned the recent finding of β-hydroxy-β-methylglutarate as a constituent of flaxseed (226) and of the alkaloid dicrotaline (135) from *Crotalaria dura* or *globifera*. The C^{14} distribution in

β-methylcrotonic acid, also detected as a reaction product in the incubation of C^{14}-acetate with liver homogenate (227) or liver extracts (228), agrees with the theory that β-methylcrotonate is derived from β-hydroxy-β-methylglutarate by removal of H_2O and CO_2 (224).

The series of reactions (equations 45 to 47) discovered by Bachhawat *et al.* (203) could explain all these findings. It would also be in accord with the observation that rat liver promotes the incorporation of acetoacetate into cholesterol (229 to 232). But until now the proof that reaction 47 is reversible and that β-hydroxy-β-methylglutaryl-CoA may be synthesized from free acetoacetate and acetyl-CoA is lacking. This pattern is also not compatible with recent findings of Blecher & Gurin (202, 233) according to which in cholesterol synthesis by rat liver slices C^{14} from 4-C^{14}-acetoacetate and from 1-C^{14}-acetate is used in the proportion 1.14/1. In the transfer of acetoacetate to β-methyl-crotonyl CoA the methyl carbon would remain in the molecule but the carboxyl carbon would be eliminated. The observation that acetate to a greater extent than acetoacetate is used for the synthesis of cholesterol (229) could also not be explained by the scheme of Bachhawat *et al.* Solution of these problems must await further experiment. At any rate experiments with isotopes do not exclude the possibility that acetoacetyl-CoA (4), instead of free acetoacetate, is used in the synthesis of β-hydroxy-β-methylglutaryl-CoA.

Final remarks.—I thank Miss Luise Wessely for her valuable assistance in the choice of literature for review. The following fields had to be excluded for lack of space: Digestion and resorption of lipides, essential fatty acids, lipoxidase, and the lipotropic factors. Furthermore, several reviews should be mentioned which can be of great value to the reader: Stadtman (234) "Studies on the Biochemical Mechanism of Fatty Acid Oxidation and Synthesis"; Greville & Stewart (235) "Fatty Acid Metabolism in Animal Tissues"; and Green (236) "Fatty Acid Oxidation in Soluble Systems of Animal Tissues."

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THE BIOCHEMISTRY OF CANCER[1,2]

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Introduction

No less than in other fields, the spate of papers in cancer research precludes any complete survey, even year by year, and many contributions of interest and importance have, perforce, been omitted from the present review. An endeavour has, however, been made to illustrate certain trends, such, for example, as the slowly but steadily growing perception of carcinogenic mechanisms, the revival of interest in tumour immunology, or the increasingly physiological interpretation of the characteristics of the cancer cell, the last reflecting the entire dependence of the subject on advances in cellular physiology in general, and in our knowledge of the unique structure and function of the nucleic acids in particular: *physiologia pathologiam illustrat.*

The cancer cell, it is commonly said, has acquired the power of unlimited growth. Twenty-five years ago, J. A. Murray, then Director of the Imperial Cancer Research Fund, pointed out that this could hardly be true *sensu stricto*, since the same power already resides in the cancer cell's normal precursor. Simple and straightforward as this comment appears, it is none the less penetrating, and it is only perhaps of recent years that we have begun to comprehend the truth behind it: that in order to explain the growth properties of the cancer cell, it is necessary to understand the growth potential of the normal cell upon which they are based, and the mechanisms—clearly of the greatest precision, orderliness, and beauty—by which this is subject to physiological regulation and control. If we are still a long way from this true understanding, hints at least are beginning to appear. Early in the history of cancer research, two main conclusions were very quickly reached from observation of the cancer cell's behaviour: first that the malignant cell and the embryonic cell are in many ways remarkably similar; and, secondly, that malignant transformation involves an essentially regressive change, a more or less pronounced simplification, or reversion to a less specialized function. Further progress had to await another two stages, first the experimental reproduction of the disease by physical and chemical means, and secondly the detailed biochemical study of the carcinogenic

[1] The survey of the literature pertaining to this review was completed in January, 1955.

[2] The following abbreviations are used: ACTH for adrenocorticotropin; ATP for adenosinetriphosphate; DNA for deoxyribonucleic acid; DPN for diphosphopyridine nucleotide; RNA for ribonucleic acid.

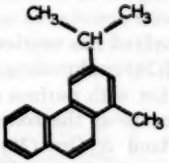
mechanism. Chemical carcinogenesis can be regarded as a very special case of the action of drugs on cells, and Ehrlich's adage applies here as elsewhere: *corpora non agunt nisi fixata*. A growing volume of work is, accordingly, being directed to the nature of the cellular substrates with which the carcinogens combine; and already there is evidence, from several different sources independently, that the combination may lead, either directly or indirectly, to the elimination of certain key proteins essential in the normal regulation of growth, thus liberating more primitive synthetic reactions upon which the process of cell division depends, and from the uncontrolled impetus of which it now proceeds more or less continuously. Here then is evidence which points, in parallel with the biological dedifferentiation of the cancer cell which has been recognized for so long, to a chemical dedifferentiation involving some loss of the mechanisms normally controlling protein synthesis. That the cell in undergoing malignant transformation seems to have passed from a less probable to a more probable state, to a state of the living system with only minimal requirements for synthesis, recalls the earlier views of Rondoni on the entropy relationships of the transformation (1), and the similar conclusions independently reached more recently by Ambrose (2). The picture is of course still very far from complete. Yet the conception of carcinogenesis through the loss of regulatory proteins, enzymes, or antigens can be regarded as a general working hypothesis of the kind always necessary in research, and without which, in Whitehead's words, no systematic thought has ever made progress. Should it be further substantiated, it may clearly prove not merely of theoretical interest but of practical importance as well, in offering the prospect of the restoration of growth control by a kind of substitutive chemotherapy; that such is not altogether beyond the bounds of possibility, is already shown by more than one analogous example in other fields.

Carcinogenesis

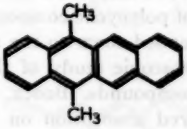
The study of carcinogenesis still remains the central approach of cancer research, and the one by which we are perhaps most likely to reach a true understanding of the essential biochemical differences between normal and malignant cells. The process can be incited by a wide and heterogeneous range of chemical and physical agents, including polycyclic aromatic hydrocarbons, azo dyestuffs, aromatic amines, biological alkylating agents, ionizing radiations, polymers and plastics, natural and synthetic oestrogens, urethane, tannic acid, and many metals; and, in certain cases, by dietary modifications, and by the induction of anaerobiosis *in vitro*. In spite of this great variety of initiating causes, certainly operating by routes which are very different primarily, it is possible, perhaps even likely, that the key change is similar in principle in every instance. Among works which have appeared during the year, and which have a particular bearing upon these problems, may specially be mentioned the welcome second edition of Greenstein's *Biochemistry of Cancer* (3), and Badger's account of chemical con-

stitution and carcinogenic activity (4), and his *Structures and Reactions of the Aromatic Compounds* (5).

The carcinogenic hydrocarbons.—In spite of the mounting emphasis on problems of mechanism of action, the past few years have nevertheless continued to see a fair number of relevant contributions in the field of organic chemistry. Thus Cason & Phillips (6) had described the synthesis of 1,9-dimethyl-1,2-benzanthracene, a compound of special interest because of its structural relationship to 3,4-benzpyrene; much earlier, Cook & Kennaway (7) had expressed serious doubt as to the prospect of ever obtaining this substance, since stereochemical relationships would necessitate the distortion out of the plane of the ring of at least one of the valence bonds between a methyl group and an aromatic ring. Wolf (8) has reported the preparation, in order to test the hypothesis of the dependence of carcinogenicity on an activated 9:10 phenanthrene double bond, of 1-methyl-3-*iso*propyl phenanthrene [I, found by Berenblum (9) to be very weakly active]; 6,11-dimethyl naphthacene (II), isomeric with the highly potent 9,10-dimethyl-1,2-benzanthracene but lacking the phenanthrene system, proved too unstable for test. Other papers include a series by Martin and his school (10), descriptions of



I



II

various halogen derivatives of 9,10-dimethyl-1,2-benzanthracene (11), and of syntheses of 2-methyl-3,4-benzphenanthrene (12), 4-methyl-1,2-benzanthracene (13), 3,4-*cyclo*penteno-1′,9-methylene-1,2-benzanthracene (14), and various sulphur isosteres of several carcinogenic hydrocarbons (15). The crystal structure of 3,4,5,6-dibenzphenanthrene has been studied by McIntosh, Robertson & Vand (16), with results of interest in relation to its biological inactivity, since the molecule departs greatly from a coplanar configuration.

As to the mechanism of action of the carcinogenic hydrocarbons, Berenblum (17) has developed his view that the process is divisible into "initiating" and "promoting" stages, and that whereas the former results in a sudden permanent change in the potentiality of the normal cell, the latter, by delaying maturation, allows a sufficient number of undifferentiated daughter cells to accumulate, and thus reach what he conceives as a colony of critical size. Other papers describe fresh examples of various inhibitory effects of the carcinogenic hydrocarbons, as of the arrest of embryonic development by conjugates of protein with 9,10-dimethyl-1,2-benzan-

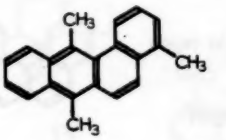
thracene (18) and the inhibition of forelimb regeneration in urodele larvae by 1,2,5,6-dibenzanthracene and methylcholanthrene (19)—effects recalling the somewhat similar inhibition of growth in various normal and tumour tissues described by the writer many years ago, produced by similar means and believed to be significant in the mechanism of carcinogenic action.

The difficult and thus far unsolved question of the intracellular site of action of the carcinogenic hydrocarbons has been further investigated by Calcutt & Payne (20), with evidence, however, that the distribution of administered 3,4-benzpyrene within mouse liver cells is extraordinarily diffuse. Setälä has summarised his studies over many years of the influence of solvents on experimental chemical carcinogenesis (21). The rate of appearance, metabolism, and disappearance of 3,4-benzpyrene in the epithelium of mouse skin, and the role of soluble proteins in the elimination of carcinogens, are the subjects of papers by Nordén (22) and Chalmers (23) respectively. In studies of the activity of the metabolic products of 3,4-benzpyrene, Cook & Schoental (24) described the phenolic metabolite 8-hydroxy-3,4-benzpyrene as feebly carcinogenic and 8-methoxy-3,4-benzpyrene as highly potent, this methyl ether of a metabolite of benzpyrene ranking among the most powerful carcinogens so far known for mice and rats. 8-Methyl-3,4-benzpyrene (25) showed activity to an extent at least equal to that of 3,4-benzpyrene itself. Boyland has continued his studies of the metabolism of polycyclic compounds [(26) naphthalene, 1-naphthol, etc.] and has also extended them to the reaction with purines and nucleic acids (27). In a spectroscopic study of the nature of the complexes of purines with aromatic compounds, Booth, Boyland & Orr (28) found that the changes in infrared absorption on formation of crystalline complexes of caffeine and tetramethyluric acid with polycyclic hydrocarbons, dibenzcarbazoles, and other polycyclic aromatic compounds, were slight but always in the same direction; the results support the suggestion that the complexes owe their formation to forces of attraction between the two components arising from their mutual polarization. The investigation by Wiest & Heidelberger (29) of the interaction of carcinogenic hydrocarbons with tissue constituents, whereby it had been shown, following topical application of 1,2,5,6-dibenzanthracene-9,10-C^{14} to the skin of mice, that irreversible chemical binding takes place between the carcinogen or its metabolite and the nucleoproteins, particulate, or soluble proteins but not with nucleic acids, has been continued. It has now been demonstrated by Heidelberger and his co-workers (30, 31) that no radioactivity is extractable, indicating covalent binding, and that essentially all the radioactive material is combined chemically not only with the original denatured protein but also with the polypeptides derived therefrom; ninhydrin treatment of the acidic material derived from the pepsin-insoluble proteins showed that one-half of the labelled metabolites were attached to amino acid residues.

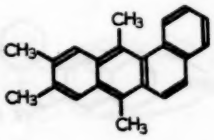
As part of a general study of the retardation of benzaldehyde oxidation, Dunn, Waters & Roitt (32) have described the actions in this regard of a

number of polycyclic aromatic hydrocarbons. Since carcinogens can act as inhibitors of biological oxidations [cf. Boyland (33)], presumably by affecting certain types of enzyme oxidase systems [cf. Rusch & Kline (34)], the possibility of correlating carcinogenic action with the inhibition of the chemical autoxidations of compounds of lipoid type had naturally been envisaged by Rusch and his colleagues. In the contribution by Waters and his school, a kinetic study of the retardation of the benzoyl peroxide-catalysed autoxidation of benzaldehyde by anthracene and by 1,2,5,6-dibenzanthracene indicated that chain termination is initially effected by combination of benzoyl-peroxy-radicals with the hydrocarbon. After longer periods of action, polycyclic hydrocarbons of different structure show differences in their type of behaviour, and classification is possible along the following lines: (*a*) hydrocarbons which retard autoxidation initially but are oxidized to products (quinones) having no such action; (*b*) initial inhibitors yielding oxidation products which are also inhibitors; and (*c*) very poor inhibitors which are oxidized to more effective inhibitors. It is of much interest that carcinogenic activity appears mainly to be associated with the hydrocarbons of group (*b*) .

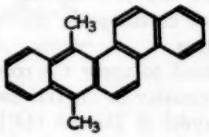
Towards a balanced assessment of the views developed over the past 10 years by the French school of theoretical physics, relating the characteristics of the active centres of the aromatic hydrocarbons to carcinogenic activity, a valuable contribution was made by Coulson (35), of which not



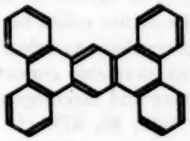
III



IV



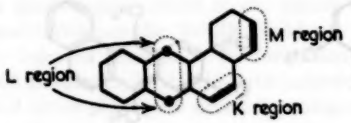
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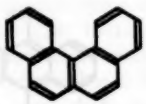
VI

the least merit is its comprehensibility not only to the general chemist but in part to the biologist as well. This subject, in relation to the general physico-chemical interpretation of the mechanism of action of carcinogenic compounds, has also been discussed by Buu-Hoï (36). In electronic delocalization studies of 4',9,10-trimethyl-1,2-benzanthracene and 6,7,9,10-tetra-

methyl-1,2-benzanthracene, (III, IV; biologically active), and of 9,10-dimethyl-1,2-(α, β) naphthanthracene (V; biologically inactive), de Clercq, Zajdela & Lehlbach [(37); cf. Pacault & Hoarau (38)] observed agreement, although not for 1,2,3,4,5,6,7,8-tetrabenzanthracene (VI) which is, however, devoid of a K region. In a report of magnetic susceptibility data for some biologically important derivatives of phenanthrene, Woernley (39) regards it as more plausible that the carcinogenic character of phenanthrene derivatives, such as methylcholanthrene, is a result of the over-all electronic configuration of the molecule, rather than that it is attributable to a specific feature such as the K region, although this may be one of a number of important determining factors. Hence, the relationship between the K region and carcinogenicity may have been weighted too heavily. A marked modification and extension of Pullman's earlier position is, however, evident in the more recent papers (40 to 43) where he develops a more complex energetic index, of which a minimum numerical value is requisite for carcinogenicity. According to this view, biological activity is in part determined not only by a favourable K region, but also by an L region (VII; of *meso*anthracene type), not so reactive as to engage the molecule in a different interaction: but Pullman also refers (42) to the exceptional case of the carcinogen 3,4-benzphenanthrene (VIII) which lacks an L region and in which the K region is insufficiently reactive. In general, the K region is selective or permissive, and the L region exclusive. Contrary to the case for addition reactions,



VII



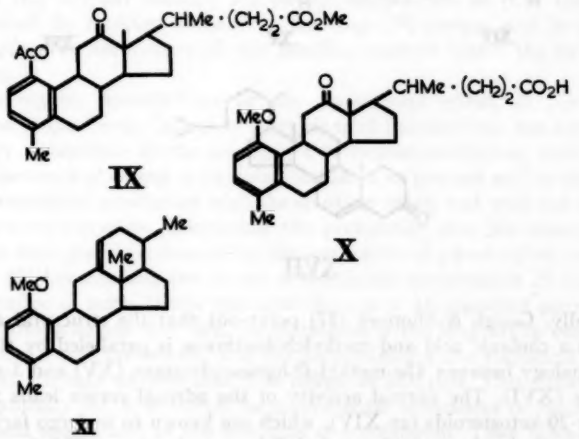
VIII

further arguments in favour of which are provided by Roux & Daudel (44), no general relation exists between carcinogenic activity and susceptibility of the hydrocarbons to substitution.

The Pullmans have also endeavoured to apply the correlation between electronic structure and carcinogenic activity to the interpretation of metabolic reactivity [(45, 46, 47); cf. Daudel & Daudel (48)] and deduce an activation towards electrophilic attack of the metabolically reactive centre of the molecule (VII; M region), calculations being in agreement with the hypothesis of perhydroxylation and the intermediary formation of an epoxide. Studies have also been made, in relation to the theory of the activated complex, of individual series such as the monomethyl benzanthracenes (49) and 3,4-benzpyrene, 3,4,8,9-dibenzpyrene, and anthanthrene (50). The latest phases of the whole subject are extensively reviewed by Pullman (51). Among cognate studies of the relation between carcinogenicity

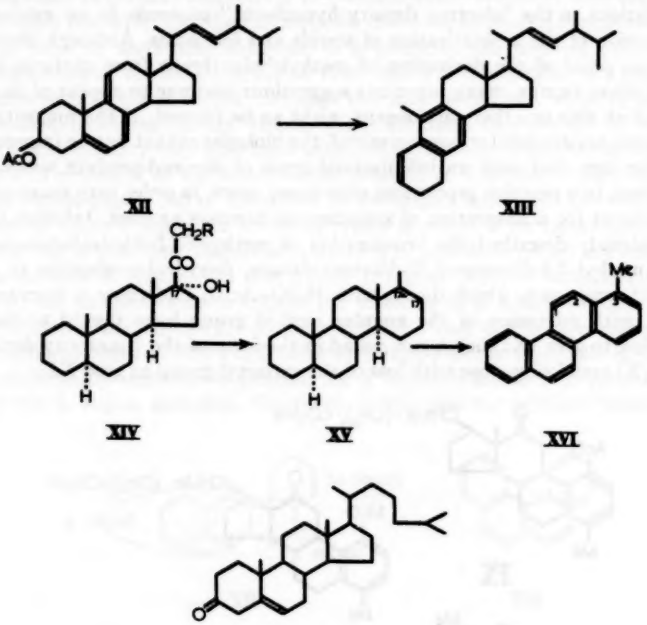
and physico-chemical properties are several by Daudel and others (52, 53) on charge distribution and basicity in the methylated angular benzacridines, those compounds with the higher pK values being the more potent biologically.

In a discussion of the relationship of natural steroids to carcinogenic aromatic compounds, Inhoffen (54), after alluding to various examples of limitations to the "electron density hypothesis," proceeds to an extensive discussion of the aromatization of sterols and bile acids. Although there is still no proof of the derivation of methylcholanthrene from cholanic acid derivatives *in vivo*, many ingenious suggestions continue to appear of routes by which this or other carcinogens might so be formed. If the majority of chemists are doubtful or noncommittal, the biologist cannot but be impressed by the fact that only an infinitesimal trace of the end-product would be required, in a reaction proceeding over many years, in order very reasonably to account for a proportion of spontaneous tumours at least. Inhoffen (55) had already described the bromination of methyl-3,12-diketocholanate to give methyl-2,4-dibromo-3,12-diketocholanate, dehydrobromination to the $\Delta^{1:2-4:5}$-compound, which diene, with H_2SO_4-Ac_2O, undergoes a rearrangement with migration of the angular methyl group from the 10 to the 4 position to give IX: such a compound in the form of the 1-methoxy derivative (X) could ring-close with loss of the carboxyl group to give XI.



A further example is seen in the Nes-Mosettig transformation (56), by the rearrangment of dehydroergosteryl acetate to a *s*-octahydroanthracene derivative. On treatment of a chloroform solution of the former (XII) with catalytic amounts of hydrogen chloride at room temperature, a skeletal rearrangement of the steroid takes place. The pure product (XIII), obtained

in a yield of about 30 per cent, lacks an oxygen function and shows an ultraviolet absorption spectrum characteristic of an aromatic ring with one conjugated double bond; the authors consider the possibility that this type of facile rearrangement, that is, the transformation of steroids into anthracene derivatives, may also conceivably be involved in spontaneous carcinogenesis.



XII XIII XIV XV XVI

XVII

Thirdly, Gough & Shoppee (57) point out that the structural analogy between a cholanic acid and methylcholanthrene is paralleled by the even closer analogy between 17*a*-methyl-*D*-homoandrostane (XV) and 3-methylchrysene (XVI). The normal activity of the adrenal cortex leads to 17α-hydroxy-20-ketosteroids (as XIV), which are known to undergo facile conversion to derivatives of 17*a*-methyl-*D*-homoandrostane (XV) by adsorption at a moist, neutral, aluminium oxide surface at 20°C. While there is no evidence that the dehydrogenation of derivatives of 17*a*-methyl-*D*-homoandrostane occurs *in vivo* as well as *in vitro*, numerous analogies can be quoted. D-Homoandrostane is not itself carcinogenic, but 3-methylchrysene appears to be weakly active.

Finally, Fieser (58) has discussed, in a similar connection, various aspects

of the chemistry and biochemistry of cholesterol. Hieger (59, 60, 61) had observed that subcutaneous injection of cholesterol in lard solution into several hundred mice resulted in a low incidence of tumours at the injection site. It appeared to Fieser that cholesterol itself could hardly have the properties of a carcinogen of even low-order potency, but that the tumours must have been initiated by some transformation product of cholesterol, or some unknown companion substance. Among the many substances derived from cholesterol by oxidation, there is, according to Fieser, only one which meets the reactivity requirements, namely, Δ^4-cholestene-3-one (XVII). The methylene group at C_4 is activated by the carbonyl group on one side and the double bond on the other, and has the same high degree of sensitivity to substitution as the *meso*-methylene group of methylcholanthrene and the *meso* nuclear position of 3,4-benzpyrene; this is, in his view, the one substance derived from or related to cholesterol most likely to possess carcinogenic activity.

Although perhaps less immediately, the biosynthesis of cholesterol itself is relevant to subsequent potential anomalies of structure and metabolism. Langdon & Bloch (62, 63) gave experimental support to the old hypothesis, [Channon (64); Robinson (65)] that squalene is an intermediate in the biosynthesis of cholesterol; the results obtained by Cornforth, Hunter & Popják (66, 67, 68) through the partial degradation of [C^{14}] cholesterol synthesised by rat-liver slices from [carboxy-C^{14}] acetate and [α-C^{14}] acetate, appear in harmony with the labelling pattern which the hypothesis requires.

Carcinogenic hydrocarbons in the environment.—Doll, in his Milroy Lectures of the Royal College of Physicians of London (69), has provided a masterly conspectus of the etiology of bronchial carcinoma, with respect to the nature and extent of its rising incidence in general, and in particular to the statistical association with the smoking habit, and with the smoking of cigarettes especially. Meanwhile the probability that the association is real, has been greatly enhanced by the first results of a forward survey [(70); and cf. (71)] comprising the causes of the deaths occurring in 29 months in a population of some 24,000 men over the age of 35, classified according to their smoking habits. The data for carcinoma of the lung are in conformity with those found previously, and not only reinforce the statistical association, but strongly suggest that it is causal in addition. This situation has very naturally led to a search for carcinogenic substances in cigarette tobacco and smoke and in the products of combustion. Thus Schmähl (72) has investigated the fluorescent substances occurring in tobacco smoke, with special reference to their retention in the lung. Cooper & Lindsey (73), using the methods of chromatography and absorption spectrophotometry, had already shown the presence, in a neutral fraction of cigarette smoke soluble in *cyclo*hexane, of anthracene and pyrene, while Commins, Cooper & Lindsey (74) demonstrated that the temperatures attained in the burning end of a cigarette fluctuate between 650° and 700°C., and hence are suffi-

ciently high to cause the pyrolysis of simpler compounds to polycyclic hydrocarbons. The same group was subsequently able to show the presence of 3,4-benzpyrene itself, and other related hydrocarbons, in both the combustion products of cigarette paper (75) and in cigarette smoke (76). In these experiments the possibility of contamination with suspended atmospheric soot was excluded by careful precautions and an exhaustive series of blank determinations. The amount of 3,4-benzpyrene in cigarette smoke was of the order of 1 μg. to every 100 cigarettes (each weighing about 1.1 gm.), and its presence was regarded as possibly attributable to the pyrolysis of acetylene. It should be noted that the effective content is rather less than that in atmospheric soot; for a person smoking 40 cigarettes per day the amount of benzpyrene drawn in during the course of a year would be approximately 150 μg. while the amount inhaled over the same period from an average urban atmosphere is *ca.* 200 μg. However, the latter is in all probability mainly adsorbed on carbon particles and may, hence, very well be relatively inactive, in contrast with the hydrocarbons in cigarette smoke which are dissolved or suspended in tiny droplets of solvent material.

In spite of the strong suspicion, or indeed virtual certainty, that the cigarette habit is responsible for at least a proportion of the recorded increase in frequency of cancer of the lung, there are few who feel confident that other environmental factors, and especially atmospheric pollution, do not also play a part. As one possible source, Kotin, Falk & Thomas (77) have demonstrated carcinogenic hydrocarbons in gasoline engine exhaust products. Of the compounds so far identified in atmospheric soot, only 3,4-benzpyrene shows marked carcinogenic properties, and in a study of polycyclic hydrocarbons in town air, Cooper (78) makes the interesting comment that cigarette smoke inhaled into the respiratory tract contains powerful solvents, such as pyridine, which may conceivably elute the carcinogens from the soot, and so render them biologically more effective. Certainly the pattern of causation may well be dual, or even, indeed, more complex.

Azo compounds.—Since the date of Badger & Lewis's review of the relationship between chemical constitution and biological activity in the carcinogenic azo compounds (79), Miller, Miller & Finger (80) have described the enhancement of activity by fluoro-substitution in 4-dimethylaminoazobenzene, concluding that no position on the prime ring is directly concerned in the carcinogenic process. The carcinogenicity of ethyl derivatives of 4-aminoazobenzene has been studied by Sugiura, Crossley & Kensler (81), a series of ethyl derivatives of this compound and of N-methyl-4-aminoazobenzene being tested in rats fed equimolar amounts in a nonprotective diet. Introduction of an ethyl group decreased or abolished activity, with the sole exception of N-methyl-4′-ethyl-4-aminoazobenzene, which proved of extreme potency in marked contrast with the low activity of N-methyl-4′-methyl-4-aminoazobenzene. An ethyl group in the 2′ position of N-methyl-4-aminoazobenzene abolished activity, whereas the 2′-methyl derivative showed low activity. Carcinogenicity was similarly decreased or abolished

by substitution of ethyl groups on the amino group. In a study of the relation of basicity and molecular dimensions to the carcinogenicity of the 4-aminoazobenzenes, Sawicki & Ray (82, 83) conclude that a planar configuration seems necessary for carcinogenicity and that substituents which distort the molecule render it inactive, a situation reminiscent of that described by the reviewer and his colleagues for the 4-aminostilbenes. They further suggest it as probable that carcinogenesis by these azo compounds involves the reaction of a base with a biological "acid."

The induction of tumours is described with 4-dimethylaminobenzene-1-azo-2-naphthalene (84) and with 1-(2-tolylazo)-2-naphthol (Oil Orange TX) (85). In further confirmation and extension of the earlier observation of the Gillmans (86) of the production of various lymphomata in rats by intermittent treatment with trypan blue, Marshall (87) reports the effect of prolonged vital staining with the same di-azo dye or with Evans' blue, in inducing a variety of proliferations of the reticular tissue varying from reversbile changes to malignant tumours. It is of interest that trypan blue is also capable of evoking abnormalities in the mouse embryo (88). Lastly, the synthesis and metabolism have been described of 4-dimethylaminoazobenzene labelled with C^{14} in the benzene rings (89).

As to the mechanism of action of the azo carcinogens, attention still centres on the hypothesis put forward by the Millers (90), and somewhat similar to that which is gradually being approached by the reviewer and his school from altogether different evidence in another connection, that the induction of malignant change is attributable to the gradual deletion of key proteins essential for the control of growth. In further chemical studies of the protein-bound dyes from rats fed 3′-methyl-4-dimethylaminoazobenzene, Brown, Miller & Miller (91) describe how crude preparations of the polar dyes, after standing for long periods in solution, gradually liberate small quantities of 3′-methyl-4-monomethylaminoazobenzene, suggesting that the polar dye may be bound to the protein through the amino nitrogen of the dye. Although the deletion hypothesis should not be accepted too uncritically, and like every other is still subject to test, many facts remain in its favour, if not necessarily all. Furthermore, it receives a certain degree of support from independent studies, such as that of Weiler (92) on the serological organ-specificity of the liver, using a fluorescence method of antigen detection in the course of carcinogenesis with butter yellow, whereby it appears that the process is accompanied by the gradual deletion of a liver antigen. Again, numerous electrophoretic studies of the soluble proteins from the livers of rats fed aminoazo dyes show first that the metabolic derivatives of these dyes are capable of binding specific liver proteins, and secondly that the soluble proteins of the supernatant tumour fractions undergo a great decrease in the lowest mobility component as compared with normal (93). Extending earlier work, Sorof, Golder & Ott (94) describe the isolation of a major class of soluble proteins from rat liver and the localization of soluble protein-bound aminoazo dyes therein. This slowest sedi-

menting class of nonparticulate proteins of rat liver was isolated by ultracentrifugation, and represents one-half of the supernatant proteins, or one-fourth of the total proteins of rat liver (3.6 component). These proteins contain the major portion of the soluble nonparticulate protein-bound aminoazo dye derivatives which result from the feeding of the hepatocarcinogens 4-dimethylaminoazobenzene or 3′-methyl-4-dimethylaminoazobenzene, the weakly active 4′-methyl-4-dimethylaminoazobenzene or, it should be noted, the noncarcinogen 2-methyl-4-dimethylaminoazobenzene. They include the "h" component, previously found to contain the bulk of the soluble protein-bound dye derivatives.

The function of the affected protein components has not as yet been defined. Obviously this is a task of the utmost importance, in particular to decide which enzymatic activities, if any, have been damaged or eliminated in the process. In general, the oxidative enzyme systems appear to be relatively deficient in induced liver tumours as compared with normal liver, although the activity of lactic acid dehydrogenase is as high as normal. On the other hand, the levels of phosphatases, nucleic acid depolymerases, cathepsins, desamidases, and nucleic acid desaminases are usually about as high in liver tumours as in normal liver (90). Decreases in liver uricase and asparaginase in the course of butter yellow carcinogenesis are reported by Mori & Shimojo (95) and by Kishi & Haruno (96) respectively. A decreased adenosinetriphosphatase activity previously observed in regenerating liver was not, however, observed by Allard & Cantero (97) in rat liver during carcinogenesis, a fall in adenosinetriphosphatase activity in the mitochondrial fraction reflecting a reduced mitochondrial population in the precancerous liver cells rather than a true enzyme depletion. A suggestion by Lamirande & Allard (98) that the nucleotide composition of the ribonucleic acid of different cell fractions is less specific in butter yellow-induced tumours than in normal rat liver is of interest, although much further work is clearly required. Ceselli & Guzzi (99) report a study of the infrared spectroscopy of liver sections in the course of carcinogenesis with butter yellow and conclude that while the nucleic acid content of the liver cells decreases during the process, that of the tumour cell proper is ultimately increased.

As is well known, the process of induction of cancer of the liver in the rat by aminoazo dyes is extraordinarily sensitive to modification by dietary and hormonal influences. In particular, Griffin, Rinfret & Corsiglia (100) demonstrated its inhibition by hypophysectomy, hepatomas being induced by 3′-methyl-4-dimethylaminoazobenzene in approximately 12 weeks in intact rats, but not even after 26 weeks in hypophysectomized rats; indeed, a pituitary-adrenal interrelationship may be a prerequisite (101). To define the role of the pituitary and adrenal, Richardson, O'Neal & Robertson (102) performed hypophysectomy at various stages during the induction period. Prior to seven weeks tumours failed to develop, but grew at later stages regardless of an adrenal atrophy manifested by lipoid depletion of the zona fasciculata. Robertson *et al.* (103, 104) then showed that the effect of hypo-

physectomy can be partially annulled by administration of ACTH, growth hormone also being moderately effective, but thyrotrophin and gonadotrophin less so. The role of ACTH in carcinogenesis which is thus suggested, was further substantiated by the discovery of the same group (105) that hypophysectomized rats injected with benzpyrene or methylcholanthrene exhibited an increased incidence of tumours when treated with ACTH simultaneously. So far as concerns the influence of hypophysectomy on tumour induction with the carcinogenic hydrocarbons, Noble & Walters (106) report findings for 9,10-dimethyl-1,2-benzanthracene somewhat in accord with the original observations of Moon, Simpson & Evans (107) in the case of methylcholanthrene. However, it appears that hypophysectomized rats are not entirely refractory to the carcinogenic action of these substances, but simply that malignant change takes place more slowly in the absence of the normal pituitary function. Similar results are presented by Ghiringhelli, Parola & Zanaboni (108) and by Agate *et al.* (109). After hypophysectomy in rats, tumours attributable to methylcholanthrene or benzpyrene appeared less frequently or more slowly, and no effect was apparent on tumours already present. Hence the hypophysis is nonessential for the induction of tumours by these means and plays no truly determining role.

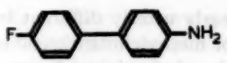
On the practical question of the safety or otherwise of the addition of colouring matters to foods, further reports have appeared by the Commission appointed for the purpose by the *Deutsche Forschungsgemeinschaft* (110, 111) and from an international meeting under the same auspices (112). A report on colouring matters, with recommendations relating to their use in foods, has also been presented by the Food Standards Committee of the British Ministry of Food (113). The main recommendation is that the appropriate public health regulations should be amended so as to permit the use in foods of specified colours only and that official approval should meantime be given, apart from certain colours of natural origin, to 32 synthetic colours, details of which are provided.

Aromatic amines.—The manufacture and use of certain dyestuff intermediates still present a hazard in the chemical industry in the form of an increased incidence of tumours of the urinary bladder. In an extended study, Case *et al.* (114) have shown that contact with benzidine, α-naphthylamine, and β-naphthylamine, in either manufacture or use, causes many more bladder tumours in workmen so exposed than would appear if no special risk was operating. Furthermore, both the onset of, and death from, these occupational tumours take place at a much earlier age than in nonoccupational cases. There is no evidence that aniline causes any increase of bladder tumours in men who handle or manufacture it, but some evidence indicates that the manufacture of magenta and auramine may cause tumours (115). β-Naphthylamine is shown to be the most potent cause of occupational tumours in Britain between 1915 and 1951, the ratios of the potencies of β-naphthylamine, mixed exposures, benzidine, and α-naphthylamine being

5.2, 2.7, 1.7, and 1.0. Tumours appear after an average induction period of 16 years for β-naphthylamine and benzidine, and 22 years for α-naphthylamine, the average induction time not being appreciably altered by the severity or duration of exposure, and hence appearing to be a feature of the causal agent. Case & Hosker (116) also describe tumour of the urinary bladder as an occupational disease in the rubber industry in England and Wales, and present findings which are consistent with, but do not prove, the hypothesis that the risk was introduced into the industry with a certain antioxidant known to have caused bladder tumours among men in the chemical industry who manufactured it, and containing about 2.5 per cent of free α- and β-naphthylamines.

In an endeavour to decipher the mode of action of carcinogenic amines generally, Clayson (117) has suggested that the process is dependent upon conversion to *o*-hydroxyamines, a conversion which is facilitated if the position *para* to the aromatic amino group is blocked to biological hydroxylation. However, much further investigation is required, and Elson (118) in particular has pointed out that while it is certainly possible that *o*-aminophenols are potentially carcinogenic, other factors, for example excretion as ethereal sulphates rather than as glucuronides (in which form are excreted the carcinogens benzidine, 4-aminodiphenyl, and 4-aminostilbene), may greatly modify their action. A recent contribution of much interest is the demonstration by Boyland and his co-workers that cancer of the bladder in man is commonly accompanied by abnormally high levels of urinary glucuronidase (119); whether this is of etiological significance or is a secondary result, still remains to be decided. The metabolism of β-naphthylamine has been studied by Henson *et al.* (120) using the compound 2-[8-C^{14}]-naphthylamine. Excretion of the radioactive compound or its metabolites into the urine or through the bile into the gut, from which they could be re-absorbed, was readily demonstrated. Excretion of 90 to 95 per cent of the radioactivity of a single dose in the urine and faeces occurs in about three days, but thereafter excretion is slow, and the compound or its metabolites remain detectable for many weeks.

Following their description of the carcinogenicity of 4-aminodiphenyl and the even more potent 3,2′-dimethyl-4-aminodiphenyl (121), Walpole, Williams & Roberts suggested that the former might be capable of causing bladder tumours in man, the presence of this base in the distillation residues of "heavy aniline," together with naphthylamines, having been suspected as partly responsible for early "aniline cancers." More recently, the authors have observed in dogs tumours of the urinary bladder produced by feeding this substance, which they describe as a more effective carcinogen in the dog than either benzidine or 2-acetylaminofluorene, and at least as potent as β-naphthylamine (122). Hyperplastic nodules in rat mammary glands, following the feeding of 4-acetamidobiphenyl, have been reported by Tuba and co-workers (123). Still more lately, another fresh observation has been made (124) of the capacity of a fluorine derivative (XVIII) to induce adenoma and adenocarcinoma of the kidney in the rat.



XVIII

Lastly, new metabolites of 2-acetylaminofluorene are described by Weisburger, Weisburger & Morris (125). The hitherto known metabolites consisted of 2-aminofluorene, 2-acetylaminofluorene, 2-amino-7-hydroxyfluorene, 2-acetylamino-7-hydroxyfluorene, and the glucuronides of the hydroxylated derivative; it is now shown that the urine of rats dosed with 2-acetylaminofluorene-9-C^{14} also contains 2-acetylamino-1- and 3-hydroxyfluorenes and their glucuronides.

Competition, interference, and synergy.—The conception put forward by Lacassagne some 10 years ago (126), of the inhibition of a given carcinogen by the simultaneous presence of a weaker agent of related chemical structure, still continues to attract interest, with the result that the reality of the phenomenon is now established and something is known of the mechanisms involved. Thus, Rudali, Buu-Hoï & Lacassagne himself have recently shown 2-aminochrysene to be an antagonist of the action of methylcholanthrene (127), and Hill *et al.* (128) have described inhibition of the development of breast tumours under the influence of 2-acetylaminofluorene by admixture of 1,2,5,6-dibenzfluorene to the diet. Experiments have also been designed to test the mutual influence of two strong carcinogens, which may result in tumour induction after a latent period longer than that required from the action of either carcinogen alone. For example, with a fixed concentration (0.1 per cent) of 9,10-dimethyl-1,2-benzanthracene, addition of increasing amounts of methylcholanthrene (0.0002 to 0.50 per cent) resulted in an increasing prolongation of the mean latent period as compared with tumours induced by 9,10-dimethyl-1,2-benzanthracene alone, but only up to a critical point [Hill *et al.* (129)]. These results are in contrast (*a*) with those obtained when the weakly carcinogenic 8-methyl-1,2-benzanthracene was added to 9,10-dimethyl-1,2-benzanthracene, when increasing concentrations of the former were associated with a constantly increasing prolongation of the latent period, and (*b*) with the converse experiment in which the concentration of methylcholanthrene was fixed (0.2 per cent) and that of 9,10-dimethyl-1,2-benzanthracene was varied (0.00004 to 0.02 per cent). Here, addition of 9,10-dimethyl-1,2-benzanthracene in the lowest concentrations had no effect, and in higher concentrations resulted in a latent period shorter than that required by methylcholanthrene alone. After a review of the literature of this topic, with reference to the possible explanation that inhibition may be a result of competition of the two substances for common receptors, Müller, Görlich & Kahofer (130) suggest that it is more probable in cases allowing mixed crystal formation, than in the case of a simple eutectic. Examples of competition and synergy have also been sought among the azo carcinogens [e.g., by Corre-Hurst *et al.* (131)], but a special case, the

mechanism of which is clearly utterly different from the above, is provided by the inhibitory action of methylcholanthrene on the induction of hepatic cancer with 3′-methyl-4-dimethylaminoazobenzene. According to Meecham, McCafferty & Jones (132), methylcholanthrene (0.0067 per cent in diet) prevented carcinoma of the liver attributable to the azo carcinogen (0.06 per cent in diet), if added to the diet before the sixth week, with partial inhibition on adding up to the tenth week but not thereafter. Pursuing the explanation of this phenomenon, Miller, Miller & Conney (133) observed that although the hepatic levels of the enzymes which cleave the azo linkage by reduction and oxidatively N-demethylate the azo dyes were greatly depressed when 0.054 per cent of 3′-methyl-4-dimethylaminoazobenzene was fed alone, the levels were nearly normal when 0.0033 per cent of methylcholanthrene was fed simultaneously. While this level did not increase these activities in the absence of dye, addition of 0.025 per cent of methylcholanthrene to the diet for two weeks, in the absence of dye, doubled the activity of each enzyme system, and 0.1 per cent of methylcholanthrene caused a five-fold increase of reductive activity. One milligram of methylcholanthrene given intraperitoneally doubled the hepatic demethylation activity of weanling rats within 18 hr. Hence, methylcholanthrene may inhibit the induction of liver cancer simply by maintaining those enzyme systems which destroy the azo carcinogen.

Biological alkylating agents.—Under this general heading can be grouped some of the latest types of chemical agent to be investigated for carcinogenic activity: the sulphur and nitrogen mustards, diepoxides, ethyleneimines, and dimethanesulphonyloxyalkanes. The dependence of their biological properties in part at least upon a certain minimum degree of chemical reactivity, recalls the earlier conviction of Fieser of a necessary association between carcinogenicity and chemical reactivity in the case of the carcinogenic hydrocarbons, and, with other properties, promises to allow a greater degree of insight into the carcinogenic mechanism than has been achieved thus far. The relation between the biological and chemical attributes of the alkylating agents has been discussed by the reviewer in detail (134), and a general account is given by Homer (135). Heston (136) describes an increased incidence of pulmonary tumours in Strain A mice exposed to mustard gas, as also (137) the occurrence of tumours in mice injected with sulphur mustard and nitrogen mustard subcutaneously. Sulphur mustard when injected subcutaneously into strains C3H, C3Hf, and A, induced neoplasms at the site, as did also the nitrogen mustard HN-2 in the two former strains. Tumours included fibrosarcomata, papillomata, squamous cell carcinoma, haemangioendothelioma, and mammary carcinoma; a significant increase was also noted in the occurrence of pulmonary tumours in strains C3H and C3Hf injected with HN-2. The occurrence of pulmonary tumours is also reported by Heston & Lorenz (138, 139) in strain A mice following whole-body x-radiation and injection of nitrogen mustard; these authors mention an inhibitory effect of irradiation upon the carcinogenic action of the mustard.

Rogers (140) describes the initiation of pulmonary adenomas in mouse lung tissue by brief exposure to nitrogen mustard *in vitro*. The reviewer's group is extending its studies to chloroethylamino derivatives of various amino acids and peptides (141, 142) and has reported (143) a remarkable contrast between the cytostatic action of the D- and L- forms of *p*-N-di(chloroethyl)-aminophenylalanine (Table I).

TABLE I

WEIGHT (GM.) OF INDIVIDUAL TUMOURS (WALKER RAT CARCINOMA) AT 13 DAYS AFTER IMPLANTATION AND 12 DAYS AFTER ADMINISTRATION OF THE D-, DL-, AND L- FORMS OF *p*-N-DI(CHLOROETHYL)AMINOPHENYLALANINE AT A DOSE OF 1 MG./KG. INTRAPERITONEALLY

Control	D-	DL-	L-
115	44	15	0.5
90	34	5	0
81	30	4	0
39	28	3	0
37	23	1	0
31	20	0	0
30	19	0	0
28	13	0	0
22	10	0	0
	6	0	0

As with the mustards, the incidence of pulmonary tumours in mice can also be increased by treatment with ethyleneimines, and Shimkin (144) reports the induction of such tumours by administration of triethylenemelamine (tri-ethyleneimino-*s*-triazine) in Strain A mice. The most substantial description of the carcinogenic action of the ethyleneimines is, however, that of Walpole *et al.* (145), who report upon several simple N-acylethyleneimines, some other monofunctional ethyleneimine derivatives, ethyleneimine itself, and β-propiolactone, as carcinogenic in rats and mice. The distribution of radioactivity following administration of triethylene imino-*s*-triazine-C^{14} in tumour-bearing and control mice has been studied by Nadkarni, Goldenthal & Smith (146) without obtaining, however, any evidence of selective localization in tumour tissue; the principal radioactive metabolite in urine was identified as cyanuric acid. Numerous papers confirm the effects of 1,4-dimethanesulphonyloxybutane ("Myleran") in the treatment of chronic myelogenous leukaemia (see below). The action of this substance on plant cells (*Pisum sativum*, *Allium cepa*) has been described by Truhaut & Deysson (147), and Burkl & Kellner (148) report the inhibition of development of the *membrana granulosa* which it is able to bring about in the course of follicular maturation.

A vital question is the nature of the cellular receptors with which the alkylating agents combine, and an excellent account of the reactions of these and other carcinogens, with macromolecules, is given by Alexander (149). From cytological evidence there is much to suggest first of all that the primary action is upon the nucleus, with functional repercussions on the cytoplasm, and secondly that the cell is most sensitive to this action during interphase, that is, during a period when the synthesis of deoxyribonucleic acid is at its maximum (150, 151, 152). Although this may clearly be more than a mere coincidence in time, nevertheless Howard & Pelc (153), from their studies of the synthesis of DNA in normal and irradiated cells, conclude that much more experimental work is needed before the possible relationships of sensitive stage, chromosome reproduction, and time of DNA synthesis can profitably be examined. Even though the action of the nitrogen mustards and related compounds is very generally attributed to interference with the synthesis of DNA, based upon morphological observation of decreased division rate and mitotic abnormality, the results of chemical analysis, and the finding of decreased incorporation of labelled precursors of nucleic acids, not all the evidence is in this direction. Mellors & Sugiura (154) report an increased content of nucleic acid per nucleus in cells of the mouse carcinoma 1025 after treatment with triethylenemelamine. However, deoxyribonucleic acid is clearly a potential substrate of unique importance, and the action upon it both of carcinogenic agents and of ionizing radiations, resulting in a breakdown of its original hydrogen-bonded structure, has been specially studied by Butler and his group (155), and by Scholes & Weiss (156, 157). The last authors describe well defined degradative chemical changes taking place when aqueous solutions of RNA and DNA are irradiated with x-rays. Fragmentation of the polynucleotides is accompanied by an increase in the number of titratable acid groups and by a liberation of inorganic phosphate and of small amounts of free purine bases. Liberation of ammonia and some ring fission of the constituent purine and pyrimidine bases occurs. Glycosidic, mono-ester, and internucleotide linkages are thus broken. The mechanism of action is via free radicals. Aqueous solutions of purines and pyrimidines and of some of their corresponding ribonucleosides and ribonucleotides were also examined. The presence of an amino group in the base, as in adenine or guanine, enhances the production of ammonia on irradiation. In both the purine and pyrimidine ring systems, however, the ring nitrogen atoms may also contribute to the ammonia yield. Chemical changes occurring on irradiation of adenosine and of yeast adenylic acid include ammonia liberation, decarboxylation, an increase in titratable acid groups, and the liberation of small amounts of free purine base. From the nucleoside some free ribose is produced, and the nucleotide suffers some dephosphorylation. Certain actions of the nitrogen mustards and of x-rays, based upon the structure suggested by Watson & Crick, are shown in Figures 1 and 2.

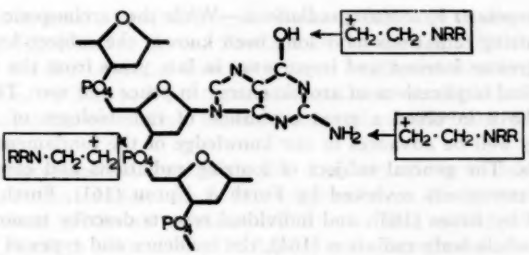


FIG. 1. Actions of nitrogen mustard.

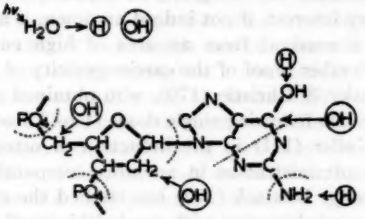


FIG. 2. Actions of x-rays.

Lastly, the Fahmys have conducted a cytogenetical analysis of the action of 1,2,3,4-diepoxybutane and of triethylenemelamine in *Drosophila melanogaster* (158, 159, 160). It is of interest that the yield of dominant lethals under the effect of the imine varies with the stage of spermatogenesis at the time of treatment; the sperm is most susceptible, so constituting a difference from the action of x-rays, to which an earlier germ mother cell is most sensitive. The diepoxybutane is characterized by high potency in the production of small deficiencies with only a small proportion of gross structural change. A special feature is the case in which the bands at the deficiency locus show various "degrees of absence," varying from defects in staining properties to a complete elimination of the band, and it is suggested that these "partial expression" deficiencies are the outcome of a modification in the molecular pattern of the gene which has prevented normal reproduction, the disabled gene reproducing itself either partially or erroneously, or not at all. The general conclusion is consistent with much other evidence, that while x-radiation induces chromosome breaks and gross structural change, the radiomimetic mutagens and carcinogens tend rather to produce fewer breaks (and consequently fewer major re-arrangements) and interfere mainly with the synthesis and reproduction of genetic material.

Carcinogenesis by ionizing radiations.—While the carcinogenic potentialities of ionizing radiations have long been known, the subject has acquired a vastly greater interest and importance in late years from the theoretical and practical implications of atomic energy in peace and war. These developments have involved a great expansion of radiobiology, of which one result may well be advances in our knowledge of the fundamentals of carcinogenesis. The general subject of ionizing radiations and carcinogenesis has been extensively reviewed by Furth & Upton (161), Furth & Lorenz (162), and by Brues (163), and individual reports describe tumours in rats following whole-body radiation (164), the incidence and types of neoplasms in x-irradiated rats following protection by post-irradiation parabiosis (165), carcinogenesis (in mice) after atomic detonations (166), pituitary tumours induced by radiothyroidectomy (167), and the development of lymphoid tumours in nonirradiated thymic grafts in thymectomized irradiated mice (168). Of cautionary interest, if not indeed an omen, is an account of osteogenic sarcoma in a muskrat from an area of high environmental radiostrontium (169). Further proof of the carcinogenicity of radiophosphorus is provided by Koletsky & Christie (170), who obtained a high incidence of sarcoma and carcinoma following single doses (1 to 3.5 μc/gm. body weight) to rats, and by Møller (171) in the induction of osteogenic sarcomas in ST/Eh mice after administration in amounts comparable with those employed therapeutically. Doniach (172) has studied the effect of radioactive iodine alone and in combination with methylthiouracil upon tumour production in the thyroid gland of the rat. He regards the danger of radio-iodine therapy as arising from the production of irreversible changes in the cells of the thyroid which render them more liable to tumour formation than are normal cells, when stimulated to undergo hyperplasia. At the same time, a dosage powerful enough to interfere with thyroxin synthesis leads indirectly to a protracted increase in the output of thyrotrophic hormone from the pituitary, which in itself constitutes a perpetual stimulus to hyperplasia of the thyroid cells. Burt, Landing & Sommers (173) provide an interesting description of the induction by radio-iodine of "amphophil" tumours of the hypophysis in mice. Histological study of the hypophyses of C57 mice suggested that the adenomas induced by large doses of I^{131} are derived from cells morphologically similar to the "thyrotrophic basophils" of rats and to the "sparsely granulated basophils" or "intermediate mucoid cells" of man. By the use of suitable fixation and staining methods, the adenoma cells could be shown to resemble not only the hypophyseal basophils with affinities for Schiff reagent, aldehyde fuchsin, or aniline blue, but also the acidophils with affinities for phosphotungstic acid, haematoxylin, and acid fuchsin. In addition, if stained with Mallory aniline blue or with haematoxylin and eosin they occasionally resemble chromophobes, and accordingly it is suggested that they be designated as amphophil adenomas. Quite apart from the carcinogenic action of radio-iodine upon the thyroid and pituitary, it is to be noted that cases have already begun to appear of

leukaemia in man consequent upon its therapeutic use in disorders of the thyroid (174). Finally, Upton, Furth & Christenberry (175) have further described the late effects of thermal neutron irradiation in mice, as qualitatively indistinguishable from those of x-radiation in the induction of leukaemia and other neoplasms, the reduction of longevity, and the induction of cataract.

Of fundamental importance are the discovery of the decisive influence of oxygen on the manifestation of such effects of x-irradiation as chromosome translocations, sex-linked recessive lethal mutations, and dominant lethal mutations in *Drosophila* (176, 177). Also of basic importance are Charlesby's studies of the effects of pile irradiation on a variety of synthetic polymers, whether by cross-linking and destruction of crystallinity (as in polyethylene and polystyrene), or by degradation as in the case of polymethylmethacrylate, these effects being measured by changes in solubility, molecular weight distribution, density, swelling properties, elastic modulus, and temperature of melting (178 to 186). These results can be achieved not only in the pile but with other sources of high energy radiation such as the linear accelerator and radioactive fission products, and in the example of a substance such as polythene they are explained as attributable to the formation of cross-links between the molecules, C-H bonds being broken by the radiation and hydrogen gas evolved. As a result, free bonds are left vacant on the carbon atoms, which unite to form a new primary bond; for polythene, polystyrene, "terylene," nylon, and unvulcanized rubber the degree of cross-linking is proportional to the radiation dose over a wide range of values (187, 188). More recently, Alexander & Charlesby (189) have studied the process of energy transfer in saturated macromolecules of different constitution exposed to ionizing radiations, and discuss the role of the process in the biological actions of radiation and its relation to the target hypothesis. Hence, although the experimental conditions in much of this work are artificial from the biological standpoint, the development is far from being without wider relevance and is a further instance of the growing importance of polymer science for biological problems.

Polymers and plastics.—Many polymers and plastics are carcinogenic in their own right, and the Oppenheimers (190) have given an account of this fascinating observation and their contributions towards it, whereby it has been established that sarcoma can be induced by implantation into the tissues of rodents of commercial regenerated cellulose (also extracted with methanol and benzene), Dacron film, "Kel-F," nylon, polyethylene (commercial and pure), polystyrene, polyvinyl chloride film, silastic (a commercial silicone product), and teflon film. In control experiments, only foreign body reactions were obtained with cotton fibres or linters as used in the manufacture of cellophane, or with surgical cotton. One tumour appeared in 50 rats implanted with chemically clean coverglasses, and Druckrey & Schmähl have now described tumours induced by the implantation of quartz (191). Of 144 tumours obtained by the Oppenheimers in

rodents, over 80 per cent were fibrosarcomas. More recently, Laskin, Robinson & Weinmann have described the production of sarcomas after implantation of methyl methacrylate (192). Oppenheimer (193) also obtained a single fibrosarcoma 313 days after embedding silk film in the tissues of an albino rat, the first instance in which a tumour may have been induced by a natural polymer. This kind of observation, and the general inertness of such polymers as polyethylene, terylene, and teflon, makes any purely "chemical" theory of action somewhat unlikely, although physical factors by themselves equally seem inadequate to provide the whole explanation. Druckrey & Schmähl (194) had postulated residual valencies on the polymer surfaces, thus enabling the latter to bind linear cell proteins, but were compelled to review this interpretation on confirming the carcinogenic action of polyethylene (195). However, they quote Hopff as showing that polyethylene can become cross-linked by oxygen, so that it may not, therefore, be as inert as is commonly supposed; mention is also made of the suggestion of Eirich, which could not however apply in the case of the carcinogenic polyamides prepared by condensation, that free radicals may be trapped during polymerisation and so be responsible for the biological action. Doubtless the explanation must await much further investigation, possibly along the lines of Danishefsky's experiments (196) using radioactive polymers, such as polymethylmethacrylate and polystyrene, labelled on the methanolic and beta carbons respectively. However, polytetrafluoroethylene films have also been found by Oppenheimer to be carcinogenic; these are completely inert, unlike polyethylene do not react with oxygen, and are in fact so chemically resistant that it is difficult to envisage any reaction into which they can enter.

Other carcinogenic influences.—Other papers concern a wide range of endocrine, dietary, and toxic agents or influences capable of inducing malignant change. Continuing their studies of endocrine carcinogenesis in the Syrian hamster, Kirkman, Robbins & Baba (197) obtained benign tumours in the *tela subcutanea* under the pigmented costo-vertebral spots, after subcutaneous implantation of pellets of stilboestrol and testosterone propionate together. Excision of the spots prior to treatment prevented tumour development, and their transplantation to the abdomen led to tumours at the new site and not at the original. These interesting tumours could be induced equally well in either sex, intact or gonadectomized, and after removal of the pellets stopped growing without, however, undergoing regression. Their appearance was not prevented by progesterone, and hypophysectomy did not retard their growth or transplantation. Following Bielschowsky's account (198) of chronic iodine deficiency as a cause of neoplasia in the thyroid of aged rats, through a compensatory increase in thyrotrophs and finally neoplastic changes in the hypophysis and long-continued stimulation by elevated amounts of thyrotrophic hormone, Axelrad & Leblond (199) also describe thyroid tumours resulting from prolonged exposure to a diet low in iodine; under treatment with iodide, certain of the changes (type α) revert, while others (types β and γ) are irreversible and represent true tumours of

the thyroid. Thyroid carcinogenesis with the goitrogen propylthiouracil is described by Van Dyke (200), while Rosin & Rachmilewitz (201) have also observed squamous cell carcinoma of the skin in rats after prolonged treatment with thiourea. The experimental development and metabolism of thyroid tumours generally are very completely reviewed by Morris (202).

As an outcome of their earlier work on the carcinogenic action of tannic acid, Korpássy and his co-workers now describe the influence of dietary protein (203), the production in rats of an experimental haemoblastosis with this agent (204), and the rapid production of malignant hepatomas by the simultaneous administration of tannic acid and 2-acetylaminofluorene (205). A fuller account of the induction of primary liver tumours in rats by treatment with a mixture of the alkaloids from *Senecio jacobaea* Linn., or with retrorsine and isatidine separately, is given by Schoental, Head & Peacock (206). Hepatic tumours have also been ascribed to prolonged feeding of ethionine (207), although in this case the tumours appear of doubtful malignancy, and Dunning & Curtis (208) have extended their observations of the relation of dietary tryptophan to the induction of tumours in the rat.

Many papers continue to confirm the specific carcinogenic action of urethane and to explore the nature of its action (209, 210). The fasting of mice for a day before and after injection of urethane was found greatly to increase the number of tumours resulting, an effect prevented by crude casein but not, however, by methionine, which, indeed, appeared further to increase the tumour yield (211). According to Malmgren & Saxén (212) the carcinogenic effect of urethane is largely confined to the first day after injection and is absent on transplanted strain A lung tissue 24 hr. after administration of urethane to the host. There persists, however, some factor which enhances the tumour incidence in urethane-treated lung tissue, transplanted into hosts previously treated with urethane, over the incidence in the same tissue transplanted into untreated animals. Rogers (213) has studied the effect upon the initiation of pulmonary adenomas in mice with urethane, of substances known to influence nucleic acid synthesis; mice given sodium deoxyribonucleate intraperitoneally a day prior to and at the time of subcutaneous injection of urethane were found to develop strikingly fewer tumours than did controls, while, contrariwise, prior treatment with aminopterin increased the tumour yield. In an investigation of the effect of known precursors of nucleic acid pyrimidines (214), orotic acid reduced the number of adenomas produced, and other results suggested the action of urethane might take place through its conjugation with another substance, possibly oxaloacetic acid or a related compound, the conjugate interfering competitively with pyrimidine synthesis at the level of orotic acid. Apart from its action in evoking pulmonary adenomas Salaman & Roe (215) have described urethane as an "initiator" (in Berenblum's specific sense) of the formation of tumours of the skin in mice.

Lastly, Hueper has continued his studies of carcinogenesis with metals (216)—injection of powdered or suspended arsenic, beryllium, or asbestos

into the femoral marrow, pleural cavity, or paranasal sinuses in rats proving, however, negative in striking contrast with the results of similar experiments with nickel or uranium (217). Sarcomata in rats following the injection of cobalt powder and the effect of cobalt on mitosis in tissue culture with reference to the phenomenon of "nucleolar persistence" have been described by Heath (218, 219).

Anaerobiosis and malignant change in vitro.—The occurrence of malignant change during the propagation of normal cells *in vitro* was recorded by Earle (220, and cf. 221, 222). Yet another example, associated however on this occasion not with chance or with the addition of a known carcinogen, is reported by Goldblatt & Cameron (223). In these experiments, inspired by the original researches of Warburg, the means used to effect the change were intermittent deprivation of air and the substitution of nitrogen during prolonged propagation. Several years before, Goldblatt & Cameron had started to explore whether, since surviving tumour cells characteristically use the glycolytic mechanism as a source of energy, the enforcement of this mechanism on growing normal cells might impose the malignant conversion. It now appears that the above technique of cultivation has twice, short of killing the cells, brought about, or been attended by, eventual malignant change; the authors accordingly suggest, although they do not claim to have proved, a causal relationship between intermittent anaerobiosis and malignant change.

Structure and Function of the Nucleic Acids

That deoxyribonucleic acid is at least a potential chemical target or substrate for the action of certain carcinogens and that the altered heredity of the cancer cell might be accounted for on some such basis, renders advances in our knowledge of the structure and function of the nucleic acids of central importance for cancer research, which is indeed to a large extent dependent upon them. Further, any problem of hereditary modification in cells must clearly be influenced by progress in our understanding of the bacterial transforming factors and their recognition as deoxyribonucleic acids affecting morphological characteristics and metabolic patterns alike (224, 225), even though there is as yet no evidence that the same principles apply to metazoan cells. Equally, progress must nevertheless depend on a critical if not indeed restrained approach, of which signs are happily by no means lacking, as for example in the comment of Skipper (226):

> It is not intended to suggest that the present fad of blaming the effects of carcinostatic, carcinogenic and mutagenic agents on their seeming disruption of nucleotide metabolism may not eventually join the "ghosts of countless once-hopeful and dazzling hypotheses." However, the evidence that certain of the heterogeneous group of agents which . . . affect neoplastic growth also affect chemical events involved in nucleotide metabolism, would seem to emphasize the necessity of attempting to obtain more basic knowledge in this field of biochemistry, if for nothing more than to lay to rest the growing assumption that here lies the core of the cancer problem.

In further studies of the role of DNA in protein synthesis, Allfrey (227) describes the incorporation of C^{14}-alanine into isolated calf thymus nuclei which proceeds only if the DNA of the nucleus remains intact. The addition of supplementary calf thymus DNA to nuclei previously treated with deoxyribonuclease resulted in higher incorporations than were observed in the absence of added DNA, recalling Gale & Folkes's account (228) of the effect of nucleic acids on protein synthesis and amino acid incorporation in disrupted staphylococcal cells. If nucleic acids act as templates in accomplishing the organization of amino acid residues within protein structures, any proof of the part they play, let alone its mechanism, must in Gale's words await an experimental system which can synthesize protein and on which an effect of nucleic acids can be demonstrated. If nucleic acid is removed from staphylococcal cells disrupted by supersonic vibration, the ability of these cells to incorporate glutamic acid and other amino acids decreases markedly but is restored by addition of nucleic acid prepared from the soluble portion of the supersonic disintegrate. The experimental facts could be fitted into a mechanism of synthesis whereby the initial structure or template is either DNA or a protein complex containing this acid, which structure is, however, not capable of the synthesis of new protein but acts as an organiser for the synthesis of RNA: once RNA has been constructed, then neo-protein synthesis can take place at a rate dependent upon the amount of RNA catalyst present. It may be that it is the RNA which collects, organises, and orients amino acid residues before they split away as protein, or that DNA combines with and organizes amino acid residues which cannot combine with a peptide chain until they have been taken over by the corresponding RNA. In a review of protein synthesis, Campbell & Work (229) critically discuss the hypothesis for peptide chain synthesis earlier proposed by Dounce (230, 231) in which RNA was chosen as the template. Accepting the suggestion that genes are composed of DNA (232), then, according to Dounce, it could conceivably happen that the DNA gene molecules would act as templates for RNA synthesis and that these ribonucleic acids would then become templates for protein synthesis in the nucleus or cytoplasm or both. However, a consideration of plasma gene and virus synthesis also leads to the concept of an independent RNA synthesis not involving nuclear genes, the relationship between plasma genes and nuclear genes being explained by the template sequence DNA→RNA→protein, with the additional postulate of a co-existing independent RNA synthesis with RNA acting as its own template. In a reply, Campbell & Work (233) point out that their objections to the template theory were not intended to suggest that nucleic acids cannot act as templates for protein synthesis, but rather to direct attention to the difficulty of any hypothesis which postulates a one-to-one relationship between genes and proteins. The biological function of DNA is further discussed by Marshak & Marshak (234). Again, even if the most widely accepted theory is that the primary genetic material is DNA or DNA-protein, and that this functions as a template, another theory is proposed which postulates that

RNA is the main genetic material, specificity at each genetic locus being determined by different types or proportions of ribonucleotides or polyribonucleotides released by the RNA-protein at that locus, alleles representing conditions at a locus that result in different rates of release of nucleotides (235). The first or orthodox hypothesis requires that DNA should be continuously present in the nucleus and chromosomes, but the Marshaks adduce evidence to suggest that it is in fact absent from the pronuclei of some echinoderm eggs. If these observations are correct, and further conviction is needed, they might invalidate the DNA-template theory, but not that according to which the primary genetic materials are indeed the ribonucleoproteins. Structural similarity between RNA and DNA is suggested by the observations that deoxyribonuclease will reduce the rate of removal of RNA nucleotides from nuclei by ribonuclease, and that after removal of its purines, DNA is digested by ribonuclease. With sufficient structural homology in the two types of nucleic acid, the Marshaks suggest competition between them. Further, since the rate of turnover of RNA *in vivo* is rapid (whereas that of DNA is very slow), DNA, where such competition exists, may well act as an inhibitor of RNA. A further critical conception is that of Chayen & Norris (236), namely, that in actively synthesizing cells, it is possible that some of the DNA is cytoplasmic; hence, it must not be treated as axiomatic that all DNA is located in the nucleus, and the assumption that the gene is necessarily composed of DNA-protein should be accepted with caution. Brown & Watson (237) discuss the heterogeneity of deoxyribonucleic acids, with reference especially to the conclusions of Chargaff and his school, and conclude that his results and their own demonstrate the DNA of a particular species to consist of a continuous distribution of molecules with varying ratios of the bases, and that the binding of these to histone increases as the ratios increase of adenine to guanine and of thymine to cytosine.

According to Franklin & Gosling (238), Stokes & Wilkins (239, 240) were the first to propose helical structures for nucleic acid as a result of direct studies of nucleic acid fibres, although a helical structure had previously been suggested by Furberg (241) on the basis of x-ray studies of nucleosides and nucleotides. Stimulated by the results and ideas of Wilkins, Stokes & Franklin, Watson & Crick (242, 243, 244) were led to propose their twin-thread complementary model as a possible structure for the paracrystalline form of the sodium salt of DNA, consisting of two DNA chains wound helically round a common axis and held together between specific pairs of bases. The pairing is highly specific, and not only must one member be a purine and the other a pyrimidine, but adenine must pair with thymine and guanine with cytosine. Since the links joining the bases to the sugar-phosphate backbone are similarly spaced in both pairs, structurally the two pairs are interchangeable. A given nucleotide can occur on either chain, but when it does, its partner on the other chain is specifically determined, thus resulting in a complementary relationship. This immediately suggests a mechanism for self-duplication, since, if we postulate that the two chains unwind and separate, then

each can serve as a template for the formation of its complement. While the bulk of the data support a hydrogen-bonded, intertwined, two-strand model of the kind proposed by Watson & Crick, the structure requires modification because of existing data, especially those pertaining to chain length. Accordingly, Dekker & Schachman (245) have suggested that a more compatible model is one in which the individual polynucleotide strands, rather than being continuous, are interrupted about every 50 nucleotides, with the breaks in the two strands staggered relative to one another—implying that the macromolecule is an aggregate of much smaller molecules held together in a specific configuration by hydrogen bonds between the purine and pyrimidine rings. Delbrück (246) also draws attention to the principal difficulty of the Watson-Crick model in the mechanism of separation of the daughter duplexes and suggests a means for resolving the locks which prevent this separation, whereby it is assumed that the complementary synthesis proceeds synchronously along the two chains and that the chains break at the growth point at every half turn of the helix, the lower terminals of the breaks immediately rejoining to the open ends of equal polarity of the new chains. The new structure, with its principle of complementary synthesis, must clearly have the most profound genetical implications, some of which have been discussed by Watson & Crick (247), and by Butler (248) in relation to the duplication of nucleotide chains and the phenomenon of crossing-over. Some critics are made doubtful from the fact that the relatively unspecific and indifferent phosphate groups are external, and the specificity shielded, while others regard the model as facile. Yet it gives some glimpse of reality, perhaps the first glimpse. Taken together, the unitary relation between the purine and pyrimidine bases in the nucleic acid structure on the one hand, and the constancy of cellular DNA on the other (249, 250), are little short of awe-inspiring, and reflect a genetical precision which, although complex in expression, in its ultimate principles must be at once simple and grand.

For many years the thought has occurred that there may be an association, in relation to tumour induction, between the planar configuration of the carcinogenic hydrocarbons and aminostilbenes in particular, and of the nucleotide plates; a reinvestigation is indicated on the basis of the new model. If carcinogenesis were indeed a kind of interference with the complementarity of synthesis based on nucleic acid, some evidences or repercussion might be expected in the nature or distribution of the nucleic acids derived from malignant as compared with normal cells. In spite of much recent work of interest (251) no such conclusion has as yet emerged clearly, although it is by no means excluded. Bader (252) examined (by absorption microspectrophotometry) the amounts of DNA per nucleus in individual cells of five different tumours and found them to be distributed into classes in the progression 1:2:4, which he interpreted as an expression of changes in chromosome content associated with polyploidy. In a somewhat similar study, Leuchtenberger, Leuchtenberger & Davis (253) found all normal tissues examined to contain cells with the same basic mean DNA content, and some tissues to

have cells with nearly exact multiple amounts; precancerous and malignant tissues showed a larger scatter from cell to cell, which, however, the authors related to the process of mitosis and did not consider a specific criterion of malignancy. In addition to these findings, Leuchtenberger, Klein & Klein (254) had earlier reported a markedly increased average RNA content per nucleus in various ascites tumours, an observation which may be similar to one made recently by Butler and his group (255), of an RNA (not removable by ordinary methods) in the "DNA" of several tumours. It may be recalled that Mauritzen, Roy & Stedman (256) reported isolated nuclei as containing small amounts of RNA, although the possibility of adsorption of cytoplasmic RNA during the isolation procedure could not be excluded. If further work is obviously required, the observation could, nevertheless, be of great importance. Khouvine (257) has also reported the DNA of several human tumours to be of variable constitution and different from that of normal DNA, and, with her group (258), has also studied the associated proteins.

Several years ago, the Stedmans indicated that the nuclei of many malignant cells differed from normal cell nuclei in containing a much smaller amount of histone. Nevertheless, deficiencies in histone did not appear to be a characteristic of cancer tissue in general. Evidence was presented for the cell specificity of histones, and the hypothesis was advanced that these basic proteins function in the nucleus as gene inhibitors, maintaining or determining the character of the cells in which they are present. Cruft, Mauritzen & Stedman (259) have now compared rat hepatoma and normal liver with similar results and with the additional finding that not only do these histones differ in physical qualities, but also in their content of lysine and arginine.

The Properties of Tumours, and Systemic Aspects

Among recent contributions to our understanding of the process of the "emergence" of the cancer cell, none is more significant than that of Furth (260) on the concept of conditioned and autonomous neoplasms, according to which tumours can be produced either through the host-environment or in the cell itself; the former are preventable and reversible, the latter irreversible and autonomous. Dependency and autonomy are recognised as relative terms, and many autonomous growths pass through a phase of dependence, the usual tendency being towards the gradual acquisition of greater independence, although complete autonomy is never certain. A related conception is that of tumour "progression," as applied by Foulds (261) especially to the evolution of cancer of the breast in mice. The subject of latent carcinoma has been specially studied by Franks (262); although latency is perhaps most frequently encountered in cancer of the prostate, it is of the utmost theoretical interest, and of some practical importance as well, in that the principle may also obtain for other tumours, as of the lung and viscera. The general question of the dormant cancer cell was the topic of Hadfield's Kettle Memorial Lecture (263). Experimentally, an interesting phenomenon has been described by Abercrombie & Heaysman (264), namely, an inhibi-

tion of cellular movement on cell contact and the diminution or absence of this effect on contact between sarcoma cells and fibroblasts; further work is, however, required.

The general question of enzyme distribution in neoplastic tissues has been considered by von Euler (265). In a study of the oxidative capacity of tumour cells and of aerobic glycolysis in homogenates of normal and tumour tissues, Reif, Potter & Le Page (266) observed a grading of oxidative capacity from high values for normal tissues to low values for tumour tissues, in a system for the aerobic glycolysis of fructose-1,6-diphosphate by tissue homogenates. Brilliant cresyl blue doubled the oxygen uptake of three out of four tumour tissues, while no effect was observed with four normal tissues. The data are interpreted in terms of a limited capacity of the DPN-cytochrome-*c* reductase in tumours. A clear-cut difference in the capacity to produce lactic acid from glucose was observed between homogenates of normal tissues and of tumours, the normal tissues being unable to produce lactic acid from glucose in the absence of other substrates, while the neoplastic tissues produced considerable quantities of lactic acid under identical conditions. As part of their study of the oxidative patterns in neoplastic tissue, directed towards an explanation of its high glycolysis, Wenner, Golder & Weinhouse (267) have investigated the complete dissimilation of glucose by slices and homogenates of normal and tumour tissues. Co-factor requirements for optimal glucose oxidation by whole homogenates of mouse tumours, determined by means of the incorporation of C^{14} into the respiratory CO_2, were here found similar to those of normal tissues; it required DPN, phosphate and magnesium, and isotonicity of incubation and homogenization media, DPN proving the most critical factor. In a hepatoma, the most active of the tumours studied, oxygen uptake was linear for 100 min. In other tumours, oxygen consumption decreased earlier but could be restored by the further addition of DPN. To test the possibility that the high glycolysis of tumour tissues might be attributable to differences from normal tissues in rates of phosphorylation or dephosphorylation, the effects of dinitrocresol on the oxidation of glucose-C^{14} were compared in both tissue types. DPN first stimulated oxidation and glycolysis in both, and inhibited at higher concentrations. The effects in homogenates were variable, but in none of the aspects of glucose catabolism tested did tumour tissues display any unique behaviour towards dinitrophenol. In a study of the levels of enzymes of the direct oxidative pathway of carbohydrate metabolism in mammalian tissues and tumours, Glock & McLean (268) found the glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities of a variety of induced and spontaneous tumours to fall within the range observed in normal tissues and to be relatively high in lymphomas and low in sarcomas. In further studies of the dynamics of the respiratory pigments of ascites tumour cells, Chance (269) verified his earlier results, there being a relative abundance of cytochrome-*c* and cytochrome oxidase; other pigments, such as flavoproteins and reduced pyridine nucleotides, were present in normal amounts. In contrast

with the low glutamyltransferase activities of most tumours examined, activities far higher than those of liver or any other normal tissue are recorded by Levintow (270) for several mouse hepatomas. Wiltshire (271, 272; cf. 273, 274) has further studied the optical form of glutamic acid in tumours. One normal tissue and four tumours were analysed for the isomers of glutamic acid by the methods previously applied to purified proteins. The percentage of D-glutamic acid formed was in all cases very small and, as in earlier work, not more than would be expected by inversion of the L-isomer during hydrolysis. Lastly, Olson *et al.* (275) record the distribution of various trace elements (zinc, molybdenum, manganese, chromium, tin, copper, nickel, aluminium, silver, lead, cobalt, iron) as between normal and malignant human liver; in all the tumour tissues these elements were markedly decreased.

Increased attention is being given to the systemic effects of tumour growth, whether produced by a secretion or stimulatory substance acting on distant tissues, or by depletion. Thus, Furth, Gadsden & Upton (276) describe transplantable ACTH-secreting pituitary tumours of mice, producing obesity, thymic atrophy, hyperglycaemia, polyuria, lymphocytopenia, and adrenal cortical hypertrophy. Hamburger and his group have continued their study of the nerve growth-stimulating factor associated with sarcomas 37 and 180, and now isolated (277). The depleting effects of transplanted tumours in rats have been further investigated by Allison, Bernstein & Babson (278; cf. 279, 280). These tumours produce a depletion similar to that from a diet deficient in protein, but with outstanding differences. Thus, the liver was depleted most rapidly of protein in rats on a protein-free diet; however, in the presence of a growing tumour, which was depleting other tissues, the protein stores of the liver remained unimpaired and even increased. Among depletion effects, the best known is the depression of liver catalase activity by a tumour fraction isolated by Greenfield & Price (281) and capable of reducing liver catalase to one-half the control value within 24 hr. Lucké & Berwick (282) have studied the quantitative relation between the growth of the Ehrlich ascites tumour and the reduction of liver catalase activity; they conclude that during the exponential phase of tumour growth the inhibitors of catalase are elaborated and released by the tumour cells at a steady rate. The influence of testosterone upon liver catalase levels in the riboflavin-deficient mouse is the subject of a report by Adams (283).

In a study of the serum electrophoretic patterns in mice, susceptible and not susceptible to mammary cancer, Johnson *et al.* (284) observed that female C3H/SP mice bearing spontaneous mammary carcinomas possessed lower values of serum gamma globulin than did controls. These low levels were also found before the onset of tumours, as early as six to seven months of age; cancer-insusceptible mice did not exhibit similar changes. In another investigation of the physico-chemical properties of crystalline human serum albumin in neoplasia, Lippincott and others (285) discovered no essential differences between normal serum albumins and those from cancer cases, by infrared spectroscopy, by electrophoresis, or immunologically.

Several papers reflect the revival of interest in the subject of cancer and the blood groups, following the indication by Aird, Bentall & Fraser Roberts (286) of such a relationship between the ABO groups and cancer of the stomach. No significant differences from the normal ABO distribution were however found by McConnell, Clarke & Downton (287) in a series of proved cases of cancer of the lung; in oat-cell tumours, an excess of group A at the expense of group O was of debatable significance. The question of blood groups and gynaecological cancer is discussed by Discombe (288).

Immunological Aspects

Recent years have witnessed a considerable revival of interest in the antigenic constitution of normal tissues and of tumours, with the advent not merely of new techniques and new facts but of fresh principles as well. Outstanding among technical developments is the rediscovery of methods allowing antigen-antibody reaction by diffusion in gels (289, 290), with additional refinements such as the detection of these interactions by interferometry (291) and the qualitative analysis of gel precipitates with the aid of colour reactions (292); and secondly the application of these methods to such problems as the antigenic constitution of normal and leukaemic leucocytes (293), of bone marrow (294), and of sea urchin hybrid embryos (295). As a further example, Korngold & Pressman (296) found antilymphosarcoma antisera, on plasma-absorbed plates, to produce five lines of interaction with lymphosarcoma extracts, absorption studies revealing three of the antigens to be shared with kidney, four with liver, lung, and spleen, and all five with lymph nodes. Other technical developments are the use of fluorescent antigens in studies of organ specificity in the course of carcinogenesis (297), already referred to, and improved methods of detecting the antibody response to tumour inoculation (298).

Among fresh facts, Mitchison (299) has reported the passive transfer of tumour immunity by the implantation of local lymph nodes from immunised mice to nonsusceptible mice; serum and peritoneal exudate were ineffective. Kidd (300) has discovered a remarkable phenomenon in the total regression of certain transplanted lymphomas, brought about *in vivo* by treatment with normal guinea-pig serum. This response is clearly open to a number of possible explanations, the validity of which is now being tested. In particular, Kidd & Todd (301) have examined the inhibitory effects of normal guinea-pig serum and immune rabbit serum on transplanted AKR lymphomas, with findings which lend weight to the supposition that iso-antibodies might be provided by the hosts to act in concert with the injected guinea-pig serum to bring about regression. The same authors (302) have also conducted experiments to decide whether iso-antibodies, capable of acting in conjunction with guinea-pig serum against 6C3HED lymphoma cells, might be present in the serum of normal mice of susceptible and resistant strains, in that of susceptible hosts carrying 6C3HED lymphomas, or in that of mice that had overcome implanted 6C3HED lymphoma cells and proved immune to re-

implantation with them. In this case the findings provide strong but not conclusive evidence that such iso-antibodies are absent from the blood of mice in all these categories. It follows, therefore, that factors of some other sort may be required to account for the participation by the host in the reaction whereby normal guinea-pig serum, injected into mice bearing 6C3HED lymphomas, brings about death of the lymphoma cells. A different phenomenon which is, however, possibly related to that of Kidd, is reported by Landschütz (303), namely, that admixture of normal human serum with cells of the Ehrlich ascites tumour, inhibits growth on subsequent injection.

Of developments which bid fair to result in new principles, none has achieved more in revolutionising our notions of tumour immunology than the study of the heterotransplantability of tumours and of the means by which resistance to the growth of foreign tumours can be virtually abolished. In great part originating in or influenced by the earlier papers of Greene, this has now reached the stage at which, by the use of cortisone, with or without x-radiation, not only human embryonic tissues such as lung, skin, cartilage, and intestine (304) but also human teratomas (305) and human malignant tumours (306, 307, 308) can be indefinitely maintained in laboratory animals and used regularly in experimental procedures. If heterologous tumours can so be induced to grow in foreign hosts, is it possible we may yet see a homologous tumour, perhaps by some immunological trick or artifice, destroyed forever by its own?

Other conceptions with profound implications for tumour immunology are Hauschka & Levan's description of the inverse correlation, marked if not entirely complete, between chromosome ploidy and host-specificity (309), and Billingham, Brent & Medawar's discovery of the principle of actively acquired tolerance to foreign cells (310), the last raising questions whether the growth of certain specific tumours, and notably perhaps chorionepithelioma, may not be a result of the same kind of immunological sensitisation. Hauschka & Levan, by a survey of lymphomas, carcinomas, and sarcomas for chromosome number, growth capacity in foreign strains, and histocompatibility genes, were able to demonstrate an influence of ploidy upon antigenicity, diploid growths being entirely strain-specific, with tetraploid tumours able to grow in foreign genotypes or even showing complete genetic indifference.

Finally, and most recently, Green (311) has presented a new conception, namely, that the immune reaction induced by a carcinogen is the essential primary stage in chemical carcinogenesis. It is postulated that the initiating stage of carcinogenesis involves the alteration of so-called "identity antigens" to iso-antigens and that the resulting antibody response either destroys the cell, or through continuous hyperplasia leads to an ever-increasing immune reaction which induces a cellular adaptation; the adaptation involves the loss of identity-protein complexes and the neoplastic cell emerges. The basic notion appears to be that all neoplastic cells lack one or more substances (probably protein complexes or enzyme systems) which normally confer on

the cell its tissue and individual specificity. The cell thus becomes antigenically more neutral and does not attract the normal immunologically regulated disposal mechanism to the same degree as before. It is argued that this lack of identity proteins constitutes the neoplastic state and that the attributes, gradations, and progressions of this state are defined by the nature and extent of the loss. Antigenic simplification or antigen absence had of course been considered many times previously as a possible cause of uncontrolled growth, but the new hypothesis can be regarded as a more detailed description of the mechanism by which such antigen deletion is brought about, and as yet another illustration of the general theory of carcinogenesis by loss. In some ways, the suggested mechanism also recalls the immunological properties of the carcinogen-protein conjugates investigated by Creech *et al.* (312, 313).

Tumours and Viruses

Although there still remains a kind of false antithesis between the chemical and infective views of cancer causation, controversy centres not so much upon the facts themselves as upon their interpretation. This is by no means likely to be simple or exclusive, in spite of an ingenious and vigorous defence of the virus hypothesis by Oberling & Guérin (314, 315)—especially since the so-called tumour viruses are heterogeneous in their nature, ranging from infective agents such as the Shope papilloma and fibroma viruses (316) through particles such as the Rous virus, with highly specific transmuting powers, to such agents as the mammary tumour inciter, which appears rather to accelerate the development or increase the number of mammary hyperplastic nodules, than to convert them to malignancy.

The relative precision of dose-response data in the tumour-virus field, as compared with that in certain other fields of biology, is considered by Bryan (317). The same author and his colleagues describe stable standard preparations of the Rous sarcoma virus, preserved by freezing and storage at low temperatures (318), and report upon the density of the virus in sucrose solutions (319). From sedimentation data, Kahler *et al.* (320) report the diameter of the Rous virus as 89 ± 3 mμ, that is, larger than previous values (65 to 75 mμ) in consequence of a density value of 1.15 instead of the older assumed value of 1.25. It is possible (319) that the density may be as low as 1.12 in dilute buffers, in which case the computed value of the diameter would increase to 100 mμ. The electron microscopy of the Rous virus continues to be difficult and inconclusive, the highly suggestive appearances which are obtained from time to time not being easily reproducible (321).

Mommaerts and co-workers (322) report a relationship between the enzymatic dephosphorylation of adenosine triphosphate and the concentration of the specific particulate component of plasma from chicks with erythromyeloblastic leukosis. The results show a proportionality during the onset of the disease which provides strong evidence that the enzymatic activity may be an intrinsic property of the particles. Also, the two are inseparable by

electrophoresis (323), and other evidence (324) also suggests that the plasma particles constitute the causative virus.

Gross (325, 326) has continued his investigation of filterable agents in Ak and C58 mouse leukaemias. In previous experiments, in order to induce the development of salivary gland carcinomas, Ak leukaemic extract (20 per cent), either ultracentrifuged or filtered, had to be inoculated into C3H mice (327). When such extracts centrifuged at 7000 g were used, the inoculated mice developed leukaemia but not salivary gland tumours. Recently however it has been found that Ak leukaemic extracts centrifuged at 7000 g (unfiltered) will also produce salivary gland carcinomas provided that the concentrations used are higher than 20 per cent. Very recently, a marked difference in susceptibility to the Ak leukaemia agent has been detected between two substrains of mice of the C3H line (328, 329). Of 162 C3H mice (National Cancer Institute) inoculated with filtered Ak leukaemic extracts only 7 developed leukaemia as compared with 90 of 320 C3H mice from another source (Bittner), the incidence of spontaneous leukaemia among 211 litter-mate controls being under 1 per cent. Nothing is known of the true nature of the Ak agent, but again it may be conjectured whether it affects not so much the leukaemic transformation directly, as susceptibility thereto.

Of obvious interest in relation to the problem of tumour viruses and non-cellular transmission is the claim of Stasney, Cantarow & Paschkis to have induced tumours by the injection of chromatin and mitochondrial fractions (330). In particular, lymphosarcomas developed in rats at the site of subcutaneous and intramedullary injection of such fractions obtained from the Murphy lymphosarcoma, while hepatomas appeared in two Wistar rats at the site of intrahepatic injection of the mitochondrial fraction of an acetylaminofluorene-induced rat hepatoma; direct proof of the absence of intact cells was impossible, but the data are regarded as indicating such contamination to be highly improbable. It will be recalled that Klein, while able to confirm the development of lymphomas after subcutaneous inoculation of chromatin fractions (331), also found that the resulting tumours did not possess the genetic constitution of the host, but that of the original tumour, the most probable explanation being contamination of the fractions with surviving cells, although such could not be detected microscopically. The whole matter is however still *sub judice*, and further data will be keenly awaited in view of its importance.

Apart from the role of viruses in the causation and propagation of tumours, the so-called oncolytic viruses continue to be the subject of much investigation. As to the mechanism of their action, Price & Ginsberg (332) produce some evidence that certain of the viruses may damage cells by direct intracellular action in the absence of viral multiplication. Moore & Diamond (333), in a study of the effect on tumour cell suspensions of such haemagglutinating viruses as those of influenza or Newcastle disease, draw attention to the similarity of the system to that of the activity of these viruses on the red blood cell. Koprowski & Love (334) describe the spectrum of viral action

in a series of ascites tumours in mice; Anopheles A and B, Bunyamwera, California, Eastern equine, Ilheus, Japanese B, louping-ill, Mengo, Murray Valley, St. Louis, West Nile, and Western equine encephalitis, as well as poliomyelitis and rabies viruses, had no inhibitory effect upon the development of the DBA and 6C3HED lymphomas or lymphoma No. 1. However, infection with Bunyamwera and Mengo viruses resulted in destruction of the Krebs 2 subline B, sarcoma 37 and the TA and MC1M tumours. The differences in response to viral infection of nine ascites tumours followed no discernible pattern with respect to histology or chromosome ploidy. In studies of the effect of viruses on human neoplasms in tissue culture, Pollard, Bussell & Snyder (335) observed St. Louis encephalitis to cause complete destruction of six of ten such explants, while the oncolytic effect of Lansing poliomyelitis virus and of neurotropic influenza A virus was incomplete, and rabies and yellow fever viruses appeared to stimulate. Other papers describe the effects on tumour cells of Rift Valley fever virus (336) and a neurotropic influenza strain (337), the mechanism of regression (338), and the multiplication of herpes, pseudorabies, vaccinia, and encephalitis viruses in the malignant human epithelial cell strain HeLa (339). Intriguing as is the phenomenon in general, it seems doubtful whether it is likely to be applicable in practice; in a pathological study of 57 human cases so treated, Newman & Southam (340) found no gross or microscopical changes attributable to any of the viruses used.

Endocrine Therapy

Although much remains to be done and opinions still differ widely, there is perhaps a trend toward a fuller understanding of the biochemical and pathological factors determining the response of mammary cancer to oestrogens, androgens, and adrenalectomy. In five of seven postmenopausal women with mammary cancer, Smith & Emerson (341) observed the excretion of excessive amounts of oestrogen in the urine, as compared with eight normal postmenopausal women. Cortisone therapy lowered these titres and reduced them to normal in three of four so treated; contrariwise, oestrogen titres rose with advancing disease. Huggins and his school (342, 343) have continued their studies of the endocrine treatment of cancer of the breast, with special reference to the characteristics of adrenal-dependent tumours. According to Huggins, four criteria have been helpful in the selection of cases for adrenalectomy, namely, age—the greatest relief being obtained in patients of 40 to 65 years; the interval between mastectomy and recurrence—improvement often following adrenalectomy when this interval is more than a year; a titre of oestrogenic substances in the urine above 15 international units daily; and the nature of the tumour—adenocarcinomata responding more commonly than undifferentiated types. Pyrah & Smiddy (344) describe 22 cases of advanced mammary cancer, with metastases, who underwent bilateral adrenalectomy and oophorectomy. Of ten who improved, five had major remissions; and the findings agreed with those of Huggins and his

co-workers that tumours having an alveolar or adenomatous structure responded to adrenalectomy more favourably. From a limited experience Strode & Burgess (345) express themselves cautiously but are doubtful whether any method exists which permits the selection of those cases most likely to benefit (see also 346, 347, 348). Martinez & Bittner (349) have investigated the effects of oophorectomy, adrenalectomy, and hypophysectomy on the growth of spontaneous mammary tumours in mice. Bilateral adrenalectomy alone or combined with oophorectomy produced a complete regression of the tumour in 3.3 per cent and 3.7 per cent of the mice respectively; hypophysectomy was followed by regression in 11.5 per cent. However, all groups in which either adrenalectomy or hypophysectomy was performed had a shorter survival time than controls not subjected to operation, even in those mice showing complete regression.

Temporary remission of prostatic cancer following adrenalectomy is described by Whitmore *et al.* (350). Changes in the steroid metabolism in prostatic cancer before and after adrenalectomy have been followed by Gemzell and his co-workers (351). The daily administration of 50 mg. cortisone acetate was sufficient to keep the 17-hydroxycorticosteroids in the blood of adrenalectomized men at an almost normal level, otherwise they disappeared in 40 to 60 hr. Fishman and others (352, 353) report a new method for estimating the serum acid phosphatase, applicable to the clinical investigation of cancer of the prostate and dependent upon the inhibition of serum acid phosphatase by L-tartrate, with evidence that the greatest part of this inhibited fraction is most likely of prostatic origin.

An interesting case of well-differentiated thyroid carcinoma, presumably dependent upon thyrotrophic hormone, is described by Balme (354), in which very numerous functioning deposits in the lungs proved resistant to treatment with radioactive iodine but were suppressed by long-continued treatment with thyroxine.

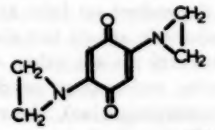
Chemotherapy

Experimental cancer chemotherapy has been extensively reviewed by Stock (355), Boyland (356, 357), and in the progress report of the Kettering-Meyer Laboratory of the Southern Research Institution (358). The special aspects of anti-metabolites and of the clinical management of the leukaemias, are dealt with by Rhoads (359), and by Burchenal (360) and Moeschlin (361), respectively. Skipper and his colleagues (362) discuss the notion of dual or sequential blocking. A series of chemical events, in which the citrovorum factor may act as a coenzyme, were examined to decide if multiple blocks might potentiate anti-leukaemic activity, the theoretical basis depending upon the use of combinations of agents which might be expected to provide for sequential or concurrent blocks in reactions leading to a single end-product. Ethionine, which is without anti-leukaemic activity alone, significantly potentiated the activity of A-methopterin. Also, the activity of A-methopterin plus 8-azaguanine was greater than that of A-methopterin alone, and

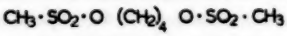
a combination of A-methopterin plus ethionine plus 8-azaguanine was more effective than any dual combination of these agents against the L1210 leukaemia. While there is no question of the interest of such effects, or of the occasional practical advantage to be gained from their application, as for example in the treatment of multiple myelomatosis with a combination of urethane and an aromatic nitrogen mustard (363), inevitably their study reflects, and is a measure of, the inadequacy of all these agents considered singly. The question has an obvious relationship to that of acquired resistance, which may be slower to develop against combinations of inhibitors than against the agents singly, although this opinion is by no means general. According to Law (364), transformations to resistance or dependence are found rather generally among acute lymphocytic leukaemias in the mouse. Leukaemic cells resistant to or dependent on folic acid analogues are inhibited by other nonrelated antileukaemic agents but show a characteristic cross-resistance (or cross-dependence) to all other 4-amino-substituted folic antagonists. Leukaemic cells, resistant to or dependent on purine antagonists (8-azaguanine or 6-mercaptopurine), show cross-resistance or cross-dependence with all other purine analogues tested, but not with other nonrelated carcinostatic agents. These changes are stable, irreversible, and heritable; experimental evidence favours the assumption that the variant cells arise by spontaneous mutation, the anti-metabolites acting as selective agents in their isolation and propagation.

Although individual members of the aromatic nitrogen mustards studied by the reviewer and his group continue to have useful if limited applications (365), their chief interest unquestionably lies in their more fundamental aspect, and in the prospect of elucidating the mechanism of their cytostatic and carcinogenic action. Of late, attention has centred upon the carboxylic derivatives of NN-di-2-chloroethylaniline (366, 367), with special reference to cyto-active amino acid and peptide derivatives (368). Bergel, Burnop & Stock (369) have described the resolution of *p*-substituted phenylalanines and the synthesis of *p*-di(chloroethyl)amino-DL-phenyl[β-C^{14}]alanine. The vastly lower biological activity of the D form of the phenylalanine mustard, as compared with the L isomer, has already been referred to (Table I) and shows a relationship similar to that obtaining for azaserine (below). The possibility was first considered (Bergel) that the D-isomer may potentially be equally active but subject to selective destruction by the higher activity of D-amino acid oxidase *in vivo* than of the L-enzyme. However, Krebs (personal communication to Professor Bergel) found neither amino acid mustard to be oxidised at a measurable rate by D-amino acid oxidase preparations, thus virtually excluding the possibility that differences in the behaviour of the D and L compounds can be attributed to selective destruction by such means. If on the other hand the differences reflect a true steric requirement, the observation may clearly provide an invaluable clue to the nature of the receptor with which the L-isomer presumably combines before exerting its characteristic action as a mustard.

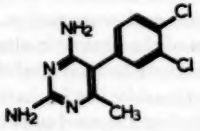
Referring to derivatives of ethyleneimine, further reports have appeared of the use of triethylenemelamine (TEM) in the treatment of lymphomas, leukaemias, and other types of neoplastic disease (370, 371), and Jackson (372) has reported resistance to this substance induced in the cells of the Walker rat carcinoma. Other examples at present under clinical trial include 2,5-*bis*ethyleneimino-1,4-benzoquinone (XIX) [Domagk, Petersen & Gauss (373)] and 1,3-*bis*(ethyleneiminosulphonyl)propane [Paterson (374)]. Schmidt compares triethylenemelamine, triethylenephosphoramide, and 6-mercaptopurine (375), while the effect of triethylene thiophosphoramide (thio-TEPA) in the treatment of chronic myelocytic chloroleukaemia in the rat is described by Shay, Gruenstein & Harris (376).



XIX



XX



XXI

Study of the clinical and other effects of Myleran (1,4-dimethanesulphonyloxybutane) (XX) (377, 378) has been continued by Galton & Till (379) with reference to its use in chronic myelogenous leukaemia, and other reports (380 to 384) are supporting. The methanesulphonic acid esters of a series of aliphatic diols, triols, and polyglycols were prepared by Carlson & Morgan (385) and tested for their ability to inhibit the growth of sarcoma 180, again with results confirming the properties of Myleran. The effects on haemopoiesis produced by Myleran on the one hand and by the carboxylic mustards already referred to, on the other, are characteristically very different, the latter operating by an SN_1 mechanism and producing a much more immediately destructive effect on the nucleus, the latter by an SN_2 mechanism and having a slower action, which also extends to the cytoplasm and is profound in the case of the platelets. It is of much interest that the two types acting together closely simulate the action of x-rays (386), thus raising the question whether the latter may be the result of two components, corresponding with the chemical methods of action described.

The clinical and experimental effects of the metabolic antagonist 6-mercaptopurine have been extensively discussed and appraised in a symposium of the New York Academy of Sciences (387). Skipper *et al.* (388) describe the substance as a moderate inhibitor of sarcoma 180, adenocarcinoma E 0771, the leukaemia L 1210, and a powerful inhibitor of adenocarcinoma 755, while its distribution and incorporation in the tissues of the mouse are recorded by Hitchings, Elion & Bieber (389). A derivative, namely 2-amino-6-mercaptopurine or thioguanine, is also described as inhibiting sarcoma 180; while more potent than 6-mercaptopurine, it is at the same time more toxic (390).

Combination chemotherapy with 8-azaguanine and a noncarcinostatic riboflavin analogue, [6-chloro-9-(1′-D-sorbityl)-*iso*alloxazine; flavotin], had previously been shown to produce greater inhibition than 8-azaguanine alone. It is now postulated by Dietrich & Shapiro (391) that flavotin potentiates the carcinostatic action of 8-azaguanine by inhibiting xanthine oxidase, thereby indirectly inhibiting guanase through product inhibition, and in turn preventing the deamination of 8-azaguanine into the inactive 8-azaxanthine. From a study of the incorporation of 8-azaguanine into the nucleic acids of tumour-bearing mice, Mandel, Carló & Smith (392) conclude that the biological effect of the antagonist is not attributable to simple replacement of preformed guanine, but rather to its action as an antimetabolite in nucleic acid metabolism after incorporation into nucleic acids. It may be recalled that Bennett, Skipper & Law (393) found the amount of 8-azaguanine incorporated into the DNA and RNA of azaguanine-susceptible tumours to be approximately 100-fold that incorporated into dependent tumours. Prusoff, Holmes & Welch (394) now describe the biological effects of 6-azathymine, in relation to the pathways of thymine and thymidine utilization.

In a study of the *Leuconostoc* citrovorum factor in cell division, Jacobson (395) finds that the arrest of cell division caused by aminopterin cannot be overcome by folic acid even in high concentration applied beforehand, when tested directly on dividing cells *in vitro*. However, citrovorum factor, applied simultaneously, prevents arrest in metaphase; it is suggested that aminopterin acts by replacing the citrovorum factor, and that the latter is essential for the step from metaphase to anaphase, that is, for the parting of the chromosome halves. Leukaemic cells from an acute mouse leukaemia were found to be powerful inactivators of aminopterin when allowed to act for 24 hr., normal bone marrow being in this respect inactive.

As competitive analogues of folic acid in the growth of *L. casei*, compounds containing the 2,4-diamino-pyrimidine moiety, including several with anti-malarial activity, are also of potential interest in the treatment of leukaemia, and accounts have now been given of their haematological and clinical effects (396, 397). In particular, 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (DDMP) (XXI) is one such antagonist of the folic acid system. In man it produces toxic effects similar to those of the 4-amino analogues of folic acid, principally bone marrow depression with megaloblastosis,

mouth ulceration, digestive disturbances, and rash. In 3 of 12 children with acute luekaemia it produced haematological improvement, but was ineffective in those resistant to A-methopterin.

Following the isolation of "azaserine" from broth-culture filtrates of a *Streptomyces*, its characterization as 0-diazoacetyl-L-serine, and the description of its tumour-inhibitory properties (398, 399), further studies have been made of its microbiology (400) and mechanism of action (401, 402), from which it appears to be a powerful inhibitor of amino acid synthesis in *Escherichia coli*, and of formate incorporation into the nucleic acids of various normal and tumour tissues. Its clinical application seems, however, to have been disappointing. Thus, Ellison and co-workers (403) describe the therapeutic responses in 41 adults and 15 children as inconstant and limited; while there were suggestions of beneficial results in Hodgkin's disease, chronic lymphatic leukaemia, and acute leukaemia in children, these usually proved to be fleeting. Attention may also be called to brief preliminary reports of the action of another antibiotic substance, namely actinomycin C, in Hodgkin's disease and various haemopathies (404).

A recent discovery of much interest is the apparent capacity of vitamin B_{12} very markedly to increase the frequency of spontaneous regression (normally about 1 to 2 per cent) in cases of neuroblastoma in children (405), an effect which, somewhat surprisingly, does not appear to depend upon increased maturation. Clearly this observation requires confirmation, and will then deserve much fuller investigation, in relation to our growing knowledge of the complex chemistry of the vitamin itself. In the last connection, Brown & Lester Smith (406) report the presence in two B_{12} vitamins of the purines 2-methyladenine and 2-methylhypoxanthine, not previously found in nature.

Among other agents with potential applications in specific forms of the disease, may be included the antimitotic *Colchicum* alkaloid "Demecolcin" in the treatment of chronic myelogenous leukaemia (407, 408), and *p*-aminobenzoic acid in the therapy of lymphoblastoma cutis and mycosis fungoides (409).

Conclusion

In concluding, some further reference may be made to the general theme of carcinogenesis by genetic, protein, enzyme, or antigen loss—a theme which tends to recur more insistently, in one or other of its forms. Functional loss or simplification in the cancer cell has of course long been known, but new examples continue to appear, as in Burnett, Anderson, Gadsden & Furth's quantitative studies of the thyroid-stimulating hormone activity of normal pituitaries and the pituitary tumours of mice, consistent with the rule that the secretions of neoplasms diminish with the acquisition of autonomy (410). If specific chemical evidence of the precise nature of the loss is less easy to obtain, allusion has already been made to the Millers' hypothesis of protein deletion in hepatic carcinogenesis, to the support which is given it by the experiments of Weiler, and to the production of genetic deletion by

the alkylating carcinogens at least. The general topic is reviewed in a stimulating fashion by Rusch (411). From the standpoint of enzymatic constitution, the picture is suggestive, with tumours having lower enzyme concentrations than most normal tissues, but only rarely showing extreme and clear-cut changes, as for example the virtual absence of azaguanine deaminase in glioblastoma in contrast with slight activity in foetal brain and high activity in adult brain (412). Moog (413) wrote: "Although the old notion that the early embryo is virtually an enzymatic blank has long since disappeared, the view that very young tissue contains only a few enzymes, and these of low activity, still seems to pervade the thinking of many chemical embryologists." Is it, however, possible that there may still be a germ of truth in this view, that the proper comparison of the cancer cell is not so much with the adult cell as with the embryonic undifferentiated cell, and that both may be driven by the abundant synthesis of growth-promoting substrates, only slowly brought under enzymatic regulation in the latter case and not at all in the former? There is increasing evidence that the growth of individual tissues and organs depends upon specific substances, as in the demonstration by Friedrich-Freksa & Zaki (414) that intraperitoneal injection of serum from partially hepatectomized rats induces mitosis in the liver of normals—recalling Aub's earlier experiments on the transmission of some such factor between rats in parabiotic union (415). As to the possible chemical nature of such substances, the writer has been impressed by the possible physiological role of xanthopterin as a stimulus to cell division in the renal tubular epithelium (416). If we speculate that specific pteridines might conceivably play a similar part in the growth of other cell types, great importance would attach to Albert's description of the transformation of purines into pteridines (417)—themselves necessary catalysts for the formation of purines—by a reaction with 1,2-dicarbonyl substances, which may shed light on pteridine biosynthesis. Further, the question would arise to what extent the action and accumulation of such substances could artificially be influenced by the enzymes, acting singly or sequentially, for which they and their products are substrates. Thus far it has not been possible to demonstrate this principle in the case of xanthopterin and xanthine oxidase, although the empirical observation has been made of a retarding action of the enzyme on the growth of spontaneous breast cancer of the mouse (418).

A somewhat related approach is apparent in recent work concerning the influence of ribonuclease on the division of amphibian eggs and of the cells of plants and tumours. Nine years ago, Thomas, Rostand & Grégoire (419) observed that puncture of undivided frog's eggs with a needle which had been dipped in a relatively pure solution of ribonuclease inhibited cell division and development—deoxyribonuclease, pepsin, and trypsin being devoid of any such action. Lately, these results have been confirmed and extended by Ledoux, Le Clerc & Vanderhaeghe (420, 421) in the case of the fertilized eggs of *Rana fusca*, *Axololl*, and *Pleurodeles*, using ribonuclease in its reduced form. The effects of the enzyme on the metabolism of living root-tip cells

have also been described by Brachet (422), where it produces marked inhibition of amino acid incorporation. The accumulation of ATP in the treated cells, as well as the fact that RNA only partly disappears after treatment with ribonuclease, are regarded by Brachet as agreeing well with Dounce's hypothesis (above) that a phosphorylated form of RNA is involved in a template mechanism of protein synthesis. Also, the effects of the enzyme on living cells are compared with those of the removal of the nucleus in unicellular organisms, that is, decrease of RNA and of protein anabolism and increased ATP, without effect upon oxygen consumption. They also recall the impairment of the uptake of C^{14}-alanine by a system of microsomes and mitocondria *in vitro*, brought about by preincubation with ribonuclease, in the experiments of Allfrey, Daly & Mirsky (423) on the role of ribonucleoprotein in protein synthesis. Results somewhat similar to those obtained by Ledoux and by Brachet are independently recorded by Kaufmann & Das (424). Treatment of growing roots of onion and lily with solutions of crystalline ribonuclease first of all stimulated mitotic activity in the polyploid cells of the region of cell elongation and modified the form and distribution of chromosomes in the meristematic cells. The production of mitotic abnormalities was associated with the degradation of ribonucleoproteins, as shown by the close correlation between the appearance of abnormalities and the modification of the staining properties of the cells with pyronin and fast green as the enzyme moved across the root; the correlation emphasizes the importance of ribonucleoproteins in determining the normal chromosome form and distribution during the course of division of meristematic cells. Lastly, Ledoux & Baltus (425, 426) have described the effects of ribonuclease upon the cells of the Ehrlich carcinoma and of various ascites tumours. Such studies of the enzymatic control of growth and cell division are of course in their merest infancy, and the outcome cannot be foreseen. As in every other field, cancer research is not only dependent upon a long-range strategy—in this case centred upon patient investigation of the carcinogenic mechanism—but is also affected by chance, the accidental observation, or the unanticipated simplifying principle. Which is likely to be the more decisive it is impossible to tell, yet each is complementary to the other, and both are essential in the advancement of our knowledge of the cancer cell.

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